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Determination of butyrates, a medicinal food to improve gut health – a chemical method using potassium triiodide

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Abstract

The use of food as medicine is important in both treating and preventing illnesses. "Gut-healthy foods" are thus essential as they help sustain our gut with a wide variety of beneficial bacteria. Short-chain fatty acids, e.g., butyrates, are a major class of metabolites produced by our gut microbiome. They play an important but incompletely understood role in many human diseases. Currently, methods like the HPLC and GCMS are most commonly used for the detection of butyrates. These methods, although popular, require specialized instruments and skilled professionals with specialized training to operate. The specialised instruments are also expensive to acquire and maintain. Therefore, there is a need for a cheaper and reliable detection method for butyrates. Hence, in this study, a protocol was developed using an enzymatic reaction coupled with a chemical and colourimetric method to detect and quantify butyrates in food samples. Briefly, the substrate acetylthiocholine was replaced in the Ellman method with butyrylcholine by esterifying the butyric acids found in food samples with choline chloride. Butyrylcholinesterase was then used for the hydrolysis of butyrylcholine. The product choline thus generated was then reacted with potassium triiodide to form choline periodide, a coloured complex that is measurable, spectroscopically. This novel potassium triiodide chemical method was found to be comparable in terms of accuracy when compared to the HPLC method. Results obtained from the quantification of butyrates found in *Cassia tora*, Kailan, Kangkong and Purple Kohlrabi Microgreen using this chemical method were within 87.23%, 91.44%, 97.49% and 97.73% respectively when compared to the HPLC method. Hence, findings from this study could lead to the development of a cheaper and more reliable method for the detection of food-derived butyrates, paving the way and contributing to the identification of food sources containing butyrates.

1. Introduction

Recently, the concept of "medicinal food" has, increasingly, been gaining popularity and more and more people are subscribing to the idea of using food as medicine. Medicinal foods not only treat illnesses but can prevent them. From a nutritional perspective, certain foods, e.g., vegetables, fruits, meats, oils, or grains, contain constituents like antioxidants, phytochemicals, vitamins, minerals, fatty acids and fibre and if consumed on a regular basis, can most probably keep us healthy into old age. The foods that we eat on a regular basis, play critical roles in regulating inflammation, controlling blood sugar concentrations, maintaining cardiovascular well-being (e.g., blood pressure and cholesterol levels), supporting our digestive system to process and remove waste, etc. Bad dietary habits, e.g., nutrient deficiencies and toxicity, are associated with numerous current health issues.

One important approach we can adopt to enhance our overall health and well-being is to maintain a good balance of our gut microbiome. Most fermented and unpasteurized foods are considered "gut-healthy" as they help to regenerate a wide variety of beneficial bacteria in our gut. A major class of metabolites synthesized in the large intestine by our anaerobic gut microbiome are the short-chain fatty acids (SCFAs) (Roy *et al*., 2006). These

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SCFAs are fatty acids with chain lengths of six or fewer carbons. Human beings do not produce SCFAs directly. They are produced in the large intestine as a by-product of the anaerobic bacterial metabolism of dietary fibre. Fatty acids like acetate, propionate and butyrate constitute slightly over 80% of the total SCFAs synthesized. Their molar ratio follows a 60:25:15 pattern, respectively (Cummings and Branch, 1990). The other SCFAs are isovaleric, isobutyric, valeric, lactic, formic, and succinic acids (Baldwin, 1970). SCFAs can readily be absorbed via the gut (Cummings *et al*., 1987), comparatively high in calories (Yang *et al*., 1970), readily metabolized by the gut as well as the liver (Cummings, 1981), promote the absorption of sodium and water in the large intestine (Roediger and Rae, 1982), and nourishes the gut (Sakata, 1987; Kripke *et al*., 1989). They play an essential but incompletely understood role in many human diseases, e.g., autoimmune diabetes (Xiao *et al*., 2017), non-alcoholic liver disease (Lambertz *et al*., 2017), cirrhosis (Fukui, 2017), neurodevelopmental disorders (Alam *et al*., 2017; Kelly *et al*., 2017; Westfall *et al*., 2017), atherosclerosis (Li and Tang, 2017), vaccine response (Lynn and Pulendran, 2018), graft vs. host disease (Yoshioka *et al*., 2017), obesity (Graham *et al*., 2015), cardiovascular disease (Tang *et al*., 2017), and kidney disease (Sabatino *et al*., 2017). Dietary butyric acid can be found in dairy products like milk and cheeses. They are also present in red meat, vegetable oils, and sauerkraut.

SCFAs are the main source of energy for colonocytes. Butyrate, in particular, plays the biggest role in colonocyte metabolism; around 90% of butyrate is metabolized by colonocytes (Cook and Sellin, 1998). Butyrates are also strongly associated with gut homeostasis (Hodin, 2000). Butyrate is also known as butyric acid or butanoic acid. To date, very little has been done to fully comprehend the benefits of butyric acid on human beings. Research so far, although limited, suggests that butyric acid might be beneficial for irritable bowel syndrome, Crohn's disease, colon cancer and insulin sensitivity (Canani *et al*., 2011; Borycka-Kiciak *et al*., 2017).

Based on the tight relationship between butyrates and the development of diseases, techniques and procedures to measure butyrate levels in different food samples are important. Currently, specialised techniques like HPLC and GCMS (Huda-Faujan *et al*., 2010; Zheng *et al*., 2013; Igarashi *et al*., 2017; Miranda *et al*., 2019; Bai and Mansell, 2020; Sowah *et al*., 2020) are most widely used for the detection and quantification of butyrates. These methods, although commonly used, are not without disadvantages. Specially trained professionals with specific skills are needed to operate these instruments. Specialized instruments as well as their regular maintenance are also costly.

Singaporeans pride themselves on their unique local food culture. Eating is a favourite Singaporean pastime. Thus, exploring the use of foods commonly found in the Singaporean diet to improve the health and general well-being of our fellow citizens would turn out to be an important quest. Moreover, as Singapore is dealing with an ageing population, research in this area would be important to support the healthy ageing of our population.

Hence, this research aimed to design, develop and validate a reliable and less costly protocol using a colourimetric method to quantify butyrates in different vegetable samples commonly found in the Singaporean diet. The lipid and fatty acid content of these vegetable samples were extracted using the modified Bligh and Dyer method (developed by Shiva *et al*., 2018). Their respective extracts, containing SCFAs, which also included butyric acid that we are interested in, were then subjected to esterification under reflux with choline chloride (ChoCl) in order to obtain, in particular, the ester butyrylcholine, as one of the products. Following that, the enzyme, butyrylcholinesterase, was added to selectively hydrolyse and release the choline from the butyrylcholine esters. According to stoichiometry, since the concentration of choline produced from the enzymatic reaction should be directly proportional to the concentration of butyric acid present in the various vegetable extracts, potassium triiodide was added to precipitate the choline out of the solution in the form of a darkly coloured choline periodide (ChoI) precipitate (Barak and Tuma, 1978). After dissolving this precipitate in a suitable solvent, the resulting solution was subjected to UV detection at 365 nm. The resulting absorbance was then used to determine the concentration of choline as well as that of the butyric acid.

2. Materials and methods

2.1 Materials

Reagents such as butyric acid, 2,6-Di-tert-butyl-4-methylphenol (also known as butylated hydroxytoluene, BHT) and choline chloride, as well as the enzyme butyrylcholinesterase were purchased from Sigma-Aldrich. All other reagents and apparatus mentioned in the following methods were readily available in the chemistry research laboratory of the National Institute of Education. Tetrahydrofuran was obtained through distillation. All the vegetables were grown using hydroponics in the laboratory and vegetable samples were obtained via the hydroponics for the experiments. The HPLC system used in the experiments was from the

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Agilent Technologies 1100 series with built-in UV and visible light detectors, as well as autosamplers. The HPLC column was purchased from Omega-Scientific. It is a YMC-Triart C18 Parker Style column with a particle size of 3 µm, an internal diameter of 4.6 mm and a length of 150 mm.

2.2 Vegetable lipids extractions

A volume of 100 mL of isopropanol was prepared in a beaker and 0.01 g (0.01%) BHT was added and completely dissolved into it. Four 50 mL centrifuge tubes were each filled with 10 mL (1 volume) of the isopropanol containing 0.01% BHT. The tubes were pre-heated to around 75°C in a water bath. Next, 7 g of Kangkong with its stem and leaves, Kailan leaves, Purple Kohlrabi Microgreen leaves and *Cassia tora* leaves were cut and grounded into fine pieces before adding them to each of the four tubes containing the isopropanol solutions. The tubes were heated for another 15 mins at 75°C. In the meantime, four other centrifuge tubes each containing a 30 mL (3 volumes) mixture of chloroform (12 mL) / methanol (16.6 mL) / water (1.4 mL) in a ratio of 30 : 41.5 : 3.5 (v/v/v) were prepared. Once the sample tubes were cooled down to room temperature, the mixtures were poured into the tubes containing the vegetable samples and stirred at room temperature overnight. After that, the mixtures were filtered and stored under -22°C, awaiting for the experiments to be conducted.

2.3 Plotting of butyric acid standard concentrations curve using high-performance liquid chromatography

Butyric acid (1 M) was prepared by adding 91.41 mL of 10.94 M butyric acid into 1000 mL of deionized water. Butyric acid standard solutions with concentrations of 0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.35%, 0.40%, 0.60%, 0.80%, and 1.0% were prepared using the 1 M butyric acid with deionized water in a total volume of 10 mL solutions. The HPLC was conditioned at 30°C with a flow rate of 0.5 mL/min, an injection volume of 10 µL, and a ratio of 20% acetonitrile: 80% deionized water using a C18 column. The standard butyric acid solutions (10 µL for 0.05 – 0.40% and 5 µL for 0.60 to 1.0%) were then subjected to HPLC for 21 mins. Three repeats were done on each of the standard solutions. The HPLC peaks were then detected using UV light at 210 nm and the respective absorbances were recorded to plot a standard curve.

2.4 Determination of vegetable butyric acid concentrations using high-performance liquid chromatography

Chloroform (1.2 mL), methanol (1.66 mL), water (0.14 mL) and 1 mL of isopropanol containing 0.01% BHT mixture was prepared. A volume of 1 mL of this solution was used as a negative control and 10 µL of butyric acid (1%) added to another 1 mL of this solution was used as a positive control. After that, 5 mL of each of the vegetable extracts were filtered and collected in 15 mL falcon tubes. A volume of 500 µL of the samples was then subjected to a 2-fold dilution with 490 µL of methanol and 10 µL of 1 M butyric acid in deionized water, making up to 1% butyric acid in each of the samples. The samples were again filtered and subjected to 3500 rpm centrifugation for 10 mins. The HPLC was conditioned at 30°C with a flow rate of 0.5 mL/min, injection volume of 5 µL, a ratio of 20% acetonitrile : 80% deionized water and a C18 column. The 2-fold diluted vegetable samples containing 1% spiked butyric acid along with the negative and positive controls were then put to HPLC for 21 mins. Three repeats were done on each sample. The HPLC peaks were then recorded at 210 nm UV light and analyzed with the help of the butyric acid standard curve to obtain the butyric acid concentrations in each of the vegetable samples.

2.5 Plotting of choline periodide absorbances ratio against butyric acid standard concentration curve using potassium triiodide after esterification and hydrolysis process

Potassium triiodide (KI_3) stock solution was prepared by dissolving 15.7 g of iodine and 20 g of potassium iodide into 100 mL of deionized water. The solution was left stirring for 45 mins to allow all solutes in the solution to dissolve. Butyric acid (1 M) was made by diluting a Butyric acid stock solution of 10.94 M using 50 mL of 99% acetonitrile. A volume of 2 mL of deionized water was added to butyrylcholinesterase enzyme (BchE) to make an enzyme stock solution. Reflux was set up by preparing 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.2% ,1.4% and 1.6% of butyric acid from the 1 M butyric acid prepared earlier above in separate 100 mL round bottom flasks containing enough acetonitrile in each flask to make up a total final solution volume of 20 mL. After that, 0.2792 g of choline chloride powder was added to each of these eight solutions to make up a final concentration of 0.1 M choline chloride. The reflux was then started and maintained at around 85°C for 2 hrs on all the flasks containing varying concentrations of butyric acid and 0.1 M of choline chloride. Then, each of these solutions containing butyrylcholine esters obtained at the end of the reflux was dried using a rotary evaporator and heating mantle, before putting them into an oven at 65°C to dry off the droplets in the flask completely. The solids obtained were weighed and recorded. Next, each solid ester was dissolved completely with 20 mL of deionized water and collected in different 50 mL centrifuge tubes. BchE (10%) was

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made using deionized water in a microcentrifuge tube from the enzyme stock solution prepared earlier and 100 µL of it was added to a microcentrifuge tube (H), whereas another tube was added with 100 µL of deionized water as a control without hydrolysis of ester (NH). A volume of 200 µL of the ester solution was then added to both of the tubes with enzyme (H) and without enzyme (NH) separately. The tubes were then subjected to 37°C water bath for 2 hrs. After that, the tubes were then added with 500 µL of KI_3 stock solution and 200 µL of deionized water to make up a total volume of 1 mL. The tubes were then vortexed and allowed to rest for 5 mins before being centrifuged at 5000 rpm for 10 mins. The supernatants of the solutions in the tubes were removed without disturbing the dark precipitate, and 1 mL of tetrahydrofuran was added to the tubes and vortexed to completely dissolve the precipitate. The contents of the tubes were then subjected to 10^{-3} dilution using tetrahydrofuran, before putting them to a microplate reader in a 96-well plate to record their individual UV absorbances at 365 nm wavelength.

2.6 Esterification, hydrolysis and choline quantitative detection of vegetable extracts

A volume of 5 mL of the vegetable extract was collected using a 100 mL round bottom flask and dried using a rotary evaporator. The resultant solid was dissolved in 20 mL of 99% acetonitrile. The mixture was then spiked with 0.5% butyric acid using 1 M butyric acid stock in acetonitrile to promote esterification, and choline chloride powder was also added to the mixture to make up to 0.1 M concentration. The mixture was subjected to reflux in a water bath for 2 hrs at around 85℃ to obtain butyrylcholine ester. After reflux, the mixture was then dried using rotary evaporator and the weight of the solid containing ester after drying was recorded. A volume of 20 mL of deionized water was added to dissolve the solid and stored in a 50 mL centrifuge tube. BchE (4%) was made using 100 µL deionized water in a microcentrifuge tube from the enzyme stock solution and added into a microcentrifuge tube (H), whereas another tube was added with 100 µL of deionized water as a control without hydrolysis of ester (NH). A volume of 200 µL of vegetable ester solution was then added to both of the tubes with enzyme (H) and without enzyme (NH) separately. The tubes were then subjected to a 37°C water bath for 1 hr. After that, the tubes were then added with 500 µL of KI_3 stock solution and 200 µL of deionized water to make up a total volume of 1 mL. The tubes were vortexed and allowed to rest for 5 mins before being centrifuged at 5000 rpm for 10 mins. The supernatant of the solutions in the tubes was removed without disturbing the dark precipitate, and 1 mL of tetrahydrofuran was added to the tubes and vortexed to completely dissolve the precipitate. The contents of the tubes were then subjected to 10^{-3} dilution using tetrahydrofuran, before putting them to a microplate reader in a 96-well plate to record their individual UV absorbances at 365 nm wavelength. Steps 1 to 15 were repeated with the rest of the vegetable extracts.

3. Results and discussion

3.1 Extraction of butyric acid from vegetable samples

Earlier studies suggested that a mixture of hexane and ethanol increased the efficiency of Soxhlet extraction of microalgal total lipids, fatty acid methyl esters (FAMEs) and their individual fatty acids, in comparison to that of using hexane alone as the solvent. Apparently, ethanol is polar, thus allowing it to extract more polar lipids, whereas hexane, which is non-polar, will be able to extract triacylglycerides as well as other neutral lipids. The yield resulting from using a mixture of hexane-ethanol in the lipid extraction was shown to be approximately three times higher than when using hexane alone (Lewis *et al*., 2000; Ryckebosch *et al*., 2012). However, different ratios of the polar and non-polar solvents used were shown to have a significant impact on the yield of the lipids. According to Shen *et al*. (2009), a 1 : 1 (v : v) of hexane and ethanol resulted in less lipid yield on *Scenedesmus dimorphus* as well as *Chlorella protothecoide*. On the other hand, studies carried out by Li *et al*. (2014) showed that a 3 : 1 (v : v) ratio of hexane and ethanol mixture resulted in a significantly higher yield of lipids from microalgae cells.

In another study, a technique known as the Bligh and Dyer method was designed to extract plant lipids, including fatty acids. This method used a solvent mixture with a one-phase system, which included chloroform, methanol and water in a volume ratio of 1 : 2 : 0.8 (v : v : v). Ryu and Wang (1998) modified this said method by incorporating a hot isopropanol treatment step, in order to inhibit lipolytic enzymes, such as phospholipase D, released by the plant tissues. They then re-extracted the plant tissues twice with chloroform : methanol (2 : 1, v : v) which made the whole process more laborious and in turn resulted in limited sample throughput. According to Shiva *et al*. (2018), a more streamlined single-extraction method was developed using Arabidopsis leaves. Such a method involved quenching the plant tissues in hot isopropanol along with a mixture of solvents while excluding a solvent evaporation phase. The solvent mixture and its volume ratio were eventually optimised and determined to be chloroform : isopropanol : methanol : water (30 : 25 : 41.5 : 3.5, v : v : v : v). The plant tissues in the mixture were shaken at room temperature for 24 hrs before being filtered and treated

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with 280 µL of 600 mM ammonium acetate. They were then subjected to analyses using a mass spectrometer (Shiva *et al.*, 2018). Hence, this method developed by Shiva *et al.* (2018) was eventually adopted in this study, to extract the butyric acid present in the various vegetable samples, because it is less laborious and does not require prolonged heat treatment, thus reducing human errors.

The vegetable samples were immersed and stirred in an extraction mixture of isopropanol, chloroform, methanol, and water, for 24 hrs. According to Figures 1 – 3, the vegetable samples were observed to be completely pulverised, turning the mixture completely green in colour. The single extraction method suggested by Shiva *et al*. (2018), was implemented in this experiment, because it was less laborious in comparison to the method utilized by Ryu and Wang (1998), therefore extensive human errors could be avoided. Specifically, the method used by Ryu and Wang (1998) involved a total of 5 repeated extractions of the vegetable tissues with the same mixture mentioned above over a period of at least 5 hrs, whereas the Shiva *et al*. (2018) method only required stirring for 24 hrs without any human interventions. Furthermore, the single extraction method could effectively reduce and minimize yield loss when compared to the Ryu and Wang (1998) method, according to the paper published by Shiva *et al*. (2018), since less repeated extraction would mean less lipids and their derivatives were lost during the filtration process. Additionally, the single extraction method was found to be suitable for large-scale lipidomics applications on plants and it does not require a solvent evaporation process before mass spectrometry which could shorten the time needed to finish the protocol.

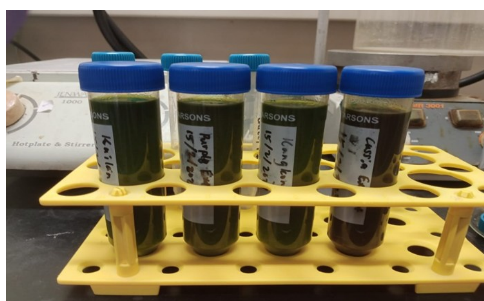

Figure 1. Unfiltered extracts of Kailan, Purple Kohlrabi Microgreen, Kangkong and *Cassia tora*.

Figure 2. Extracts of Kailan, Purple Kohlrabi Microgreen, Kangkong and *Cassia tora* being filtered into conical flasks.

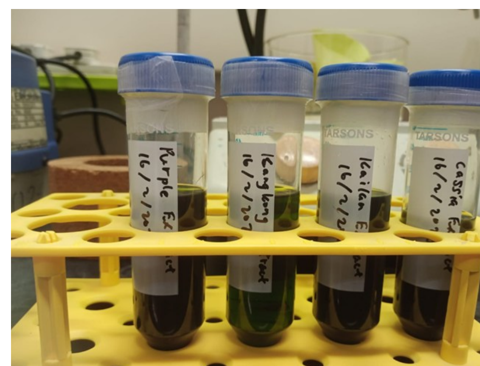

Figure 3. Filtered extracts of Kailan, Purple Kohlrabi Microgreen, Kangkong and *Cassia tora*.

A YMC-Triart C18 column was selected because of the non-polar nature of its solid stationary phase, which would help in the elution and separation of polar butyric acid from other non-polar compounds. The stationary phase consists of octyldecylsilane and has 18 carbons bound to the silica layer. Compared to similar linear alkylsilanes such as C8 and C4, C18 has a much longer carbon chain length. Due to these extra carbons, C18 possesses a larger surface area, thus increasing the interaction time of the mobile phase containing the sample with the stationary phase. As a result, the sample will elute more slowly, resulting in better separations of the HPLC peaks. Furthermore, a slower flow rate of 0.5 mL/min was also used to allow for longer elution time, thus further enhancing the resolution of the HPLC peaks. After several trial-and-error experiments, a column at a temperature of 30°C, a sample injection volume of 5 µL, and 20% acetonitrile with 80% deionized water, were found to be the optimal conditions for the separation and resolution of the HPLC peaks.

3.2 Determining butyric acid concentration using the high performance liquid chromatography method

In order to determine the accuracy of the potassium triiodide chemical method, the HPLC method was selected for comparison as it is currently the most commonly used standard method for determining butyric acid concentrations in different food samples. Thus, butyric acid standard solutions at different concentrations were prepared and their absorbances were measured using HPLC. The average absorbances (n = 3) at 210 nm of Butyric acid standard solutions at 0%, 0.05, 0.10, 0.15, 0.20, 0.25, 0.3, 0.35, 0.4, 0.6, 0.8 and 1.00% measured using the HPLC method are shown in Table 1. A Butyric acid standard calibration curve was constructed by plotting these average absorbance values against their respective standard Butyric acid concentrations (Figure 4) and a straight line with R^2 = 0.9851 was obtained. Figure 5 shows the chromatograms of the butyric acid standard calibration curve (0 - 1%)

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Table 1. Butyric acid standard solutions and their respective absorbance at 210 nm wavelength were measured using HPLC with a UV detector, with sample injection volumes of 10 µL.

Sample	Name	Concentrations (%)	210 nm (mAU)	Repeat	Average of absorbances (mAU)
SPL1	Butyric acid (0%)	0.00	0.0000 0.0000 0.0000	3	0.0000
SPL2	Butyric acid (0.05%)	0.05	4.5171 3.9592 4.0769	3	4.1844
SPL3	Butyric acid (0.10%)	0.10	4.2530 4.0922 7.5555	3	5.3002
SPL4	Butyric acid (0.15%)	0.15	5.2883 5.2873 8.6618	3	6.4125
SPL5	Butyric acid (0.20%)	0.20	6.5954 6.6890 9.4498	3	7.5781
SPL6	Butyric acid (0.25%)	0.25	7.9504 7.7823 12.4659	3	9.3995
SPL7	Butyric acid (0.30%)	0.30	9.2989 8.7856 15.2701	3	11.1182
SPL8	Butyric acid (0.35%)	0.35	10.0036 10.1913 16.8750	3	12.3566
SPL9	Butyric acid (0.40%)	0.40	11.7913 11.3965 17.9173	3	13.7017
SPL10	Butyric acid (0.60%)	0.60	19.3770 19.7325 19.5539	3	19.5545
SPL11	Butyric acid (0.80%)	0.80	24.2925 24.1696 24.2015	3	24.2212
SPL12	Butyric acid (1.00%)	1.00	27.9410 27.0197 27.6432	3	27.5346
		Mean	11.7801	Standard Deviation	8.3038

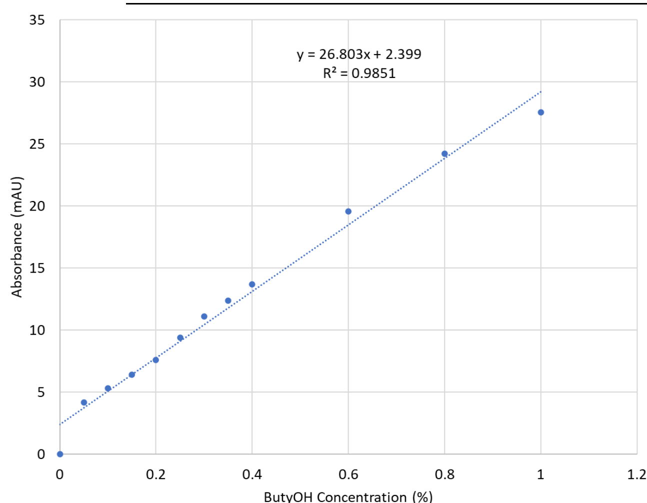

Figure 4. Butyric acid standard calibration curve obtained using HPLC showing absorbance (mAU) against butyric acid standard concentration (%).

obtained using HPLC.

After establishing the standard calibration curve to determine butyric acid concentrations using the HPLC described, the butyric acid concentrations of the four vegetable samples previously extracted as described above were determined. The average absorbances (n = 3) at 210 nm of four different vegetables (Kangkong, Kailan, Purple Kohlrabi Microgreen and *Cassia tora*) spiked with 1% butyric acid were measured using the HPLC method are shown in Table 2. Their average corrected absorbances were calculated based on the average absorbance value of 1% Butyric acid obtained. The concentrations of the spiked samples were then obtained based on the linear relationship established in the standard calibration curve (Figure 4), taking into

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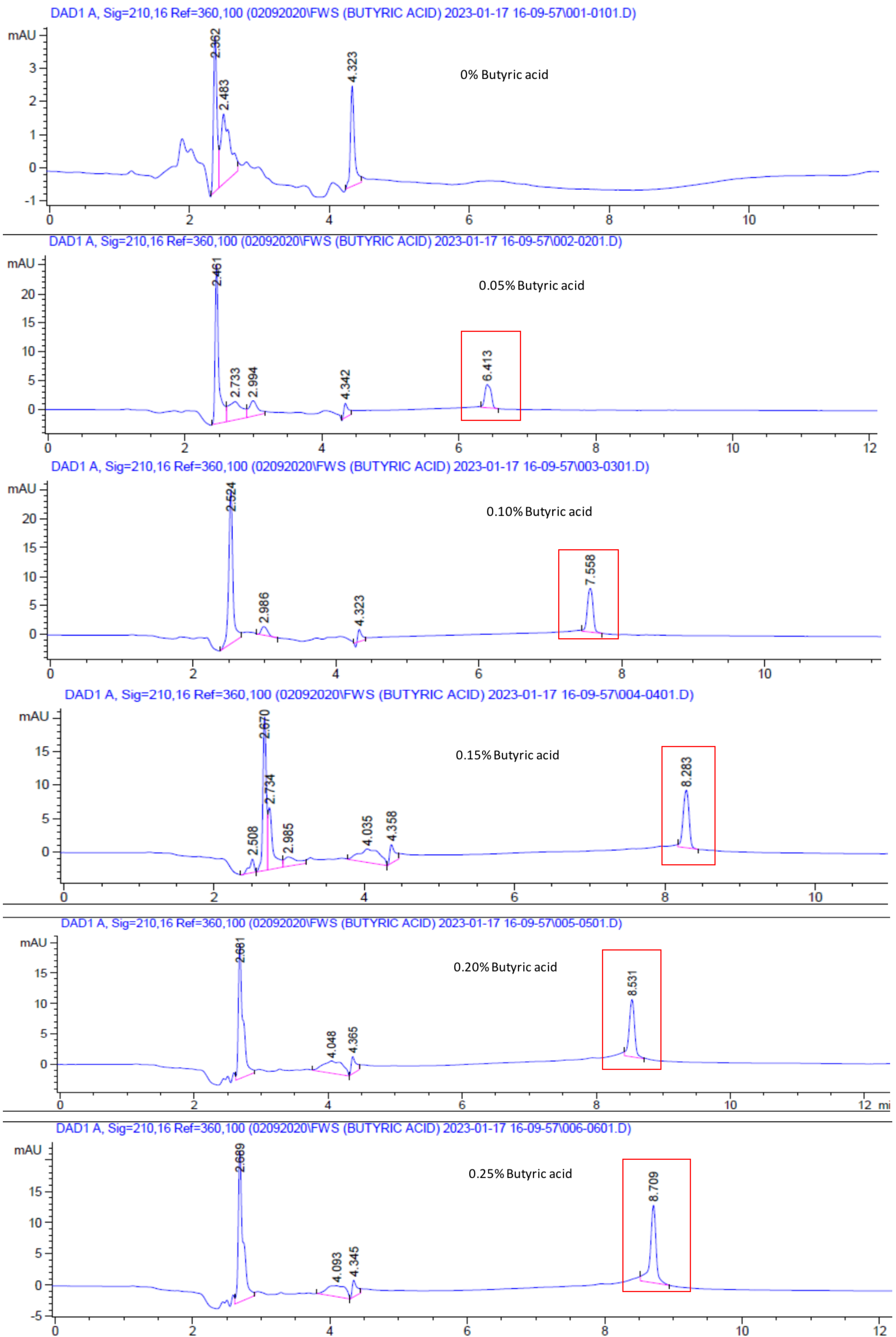

Figure 5. HPLC chromatograms of the butyric acid standard calibration curve (0 to 1%).

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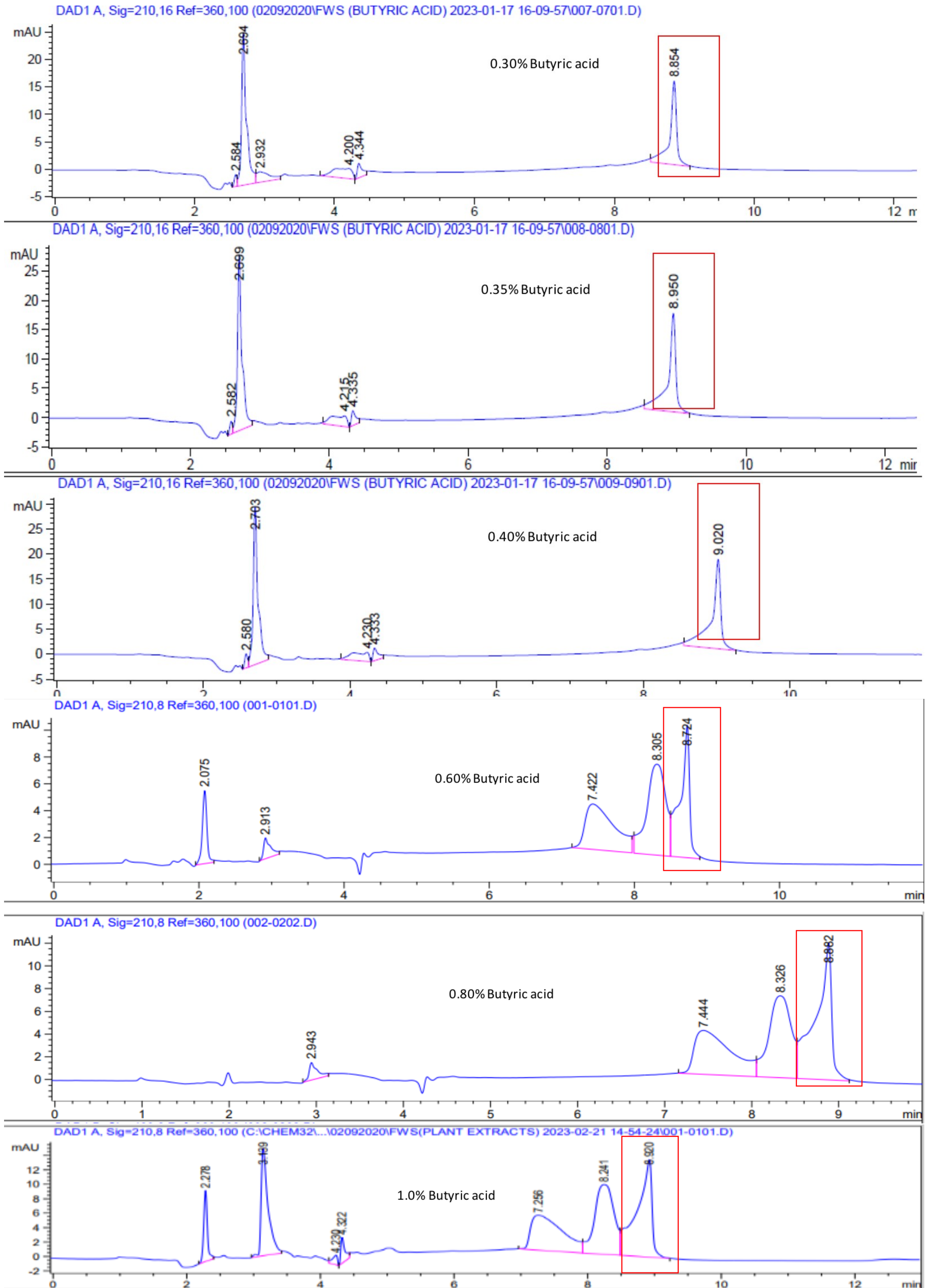

Figure 5 (Cont.). HPLC chromatograms of the butyric acid standard calibration curve (0 to 1%).

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Table 2. Vegetable extracts spiked with 1 % butyric acid solution and their respective butyric acid absorbance at 210 nm wavelength, measured using HPLC with a UV detector.

Sample	Injection Volume (μL)	Weight (g)	Dilution	Milli Absorbance Unit (mAU)	Average Corrected Absorbance at 5 μL (mAU)	Average Corrected Absorbance at 10 μL (mAU)	2-Fold Diluted Concentrations (%)	Final ButyOH Concentrations (%)
Butyric Acid (1%)	5	7	NA	13.9705 13.5098 13.8216	13.7673	27.5346	NA	1
Kangkong (L+S)	5	7	2 Fold	38.9481 39.8681 45.1672	27.5605	55.1210	1.97	3.93
Kailan (L)	5	7	2 Fold	26.9585 28.1438 35.6560	16.4855	32.9709	1.14	2.28
Purple (L)	5	7	2 Fold	95.1275 58.0418 44.5539	52.1404	104.2809	3.80	7.60
Cassia tora (L)	5	7	2 Fold	201.5804 351.1590 207.0050	239.4808	478.9616	17.78	35.56

consideration the dilution factor. Hence, using the HPLC method, the concentration of butyric acid detected was 3.93% in Kangkong, 2.28% in Kailan, 7.60% in Purple Kohlrabi Microgreen and 35.56% in *Cassia tora*.

Based on the HPLC method, results obtained indicated that amongst the four vegetable extracts studied, *Cassia tora* has the highest butyric acid concentration of 35.56%. According to Shukla *et al.* (2018), the saturated fatty acid content of *Cassia tora* was found to range from 22% to 61%. The above-mentioned fatty acid content very likely included butyric acid, hence verifying, and supporting indirectly, the validity of the butyric acid concentration of *Cassia tora* (35.56%) detected and calculated using the HPLC method in this study. Unfortunately, there are no reliabe references available to use as benchmarks in the determination of butyric acid concentrations of the other three vegetable extracts, i.e., Kailan, Kangkong and Purple Kohlrabi Microgreen. Figure 6 shows the chromatograms of the different vegetable extracts spiked with 1% butyric acid obtained using the HPLC method. It is worth noting that any future attempts to reproduce the results obtained in this study should take into account the fact that the four vegetables investigated were all grown using hydroponics in a laboratory.

3.3 Determining butyric acid concentration using the potassium triiodide chemical method

According to Appleton *et al.* (1953), in order to quantify choline, choline as a quaternary ammonium compound can be reacted with potassium triiodide to form a coloured compound, choline periodide. Its absorbance is thus measurable when dissolved in ethylene dichloride using a UV-VIS spectrometer.

The relationship between the concentrations of butyric acid and choline periodide was determined via the esterification process of butyric acid and choline chloride. Through esterification, butyrylcholine chloride and other esters were formed. Butyrylcholine chloride was then selectively hydrolysed by the enzyme butyrylcholinesterase to give butyric acid and choline chloride. Stoichiometrically, after the enzymatic hydrolysis of one mole of the substrate butyrylcholine chloride, one mole of the product choline will be formed. After that, potassium triiodide was added to the solution containing the choline. A dark choline periodide precipitate was formed with the choline that was released from the enzymatic reaction. The supernatant was then removed, and the precipitate was dissolved in tetrahydrofuran. The absorbance of the resultant solution was measured and recorded at 365 nm, which is the absorbance wavelength of choline.

Based on the relationship between the concentrations of butyric acid and choline periodide described above, a standard calibration curve was obtained using varying concentrations of butyric acid. Butyric acid standard solutions at 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6% against the average absorbances (n = 4) of choline periodide at 365 nm measured are shown in Table 3. The average corrected absorbances of choline chloride were calculated based on the average absorbance value of 0% butyric acid (tetrahydrofuran only) obtained. A choline

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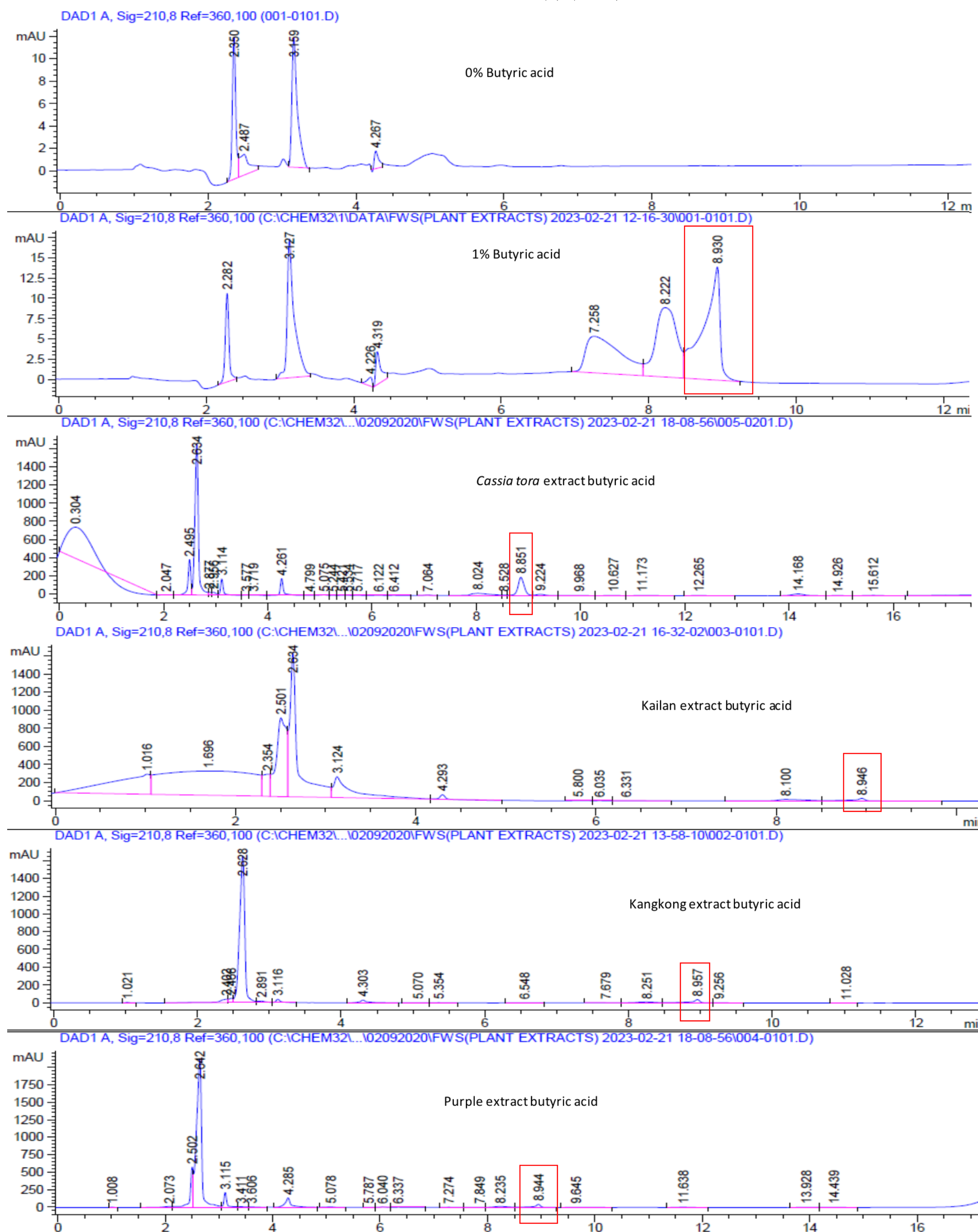

Figure 6. HPLC chromatograms of the different vegetable extracts.

periodide hydrolysed (H) and unhydrolyzed (NH) ratio (by BChE) for each concentration of butyric acid mentioned above was also calculated (Table 4) based on the respective average corrected absorbance values obtained in Table 3. A standard calibration curve was then constructed by plotting the choline periodide hydrolysed (H) and unhydrolyzed (NH) ratio against the respective standard Butyric acid concentrations (Figure 7). With increasing butyric acid concentration from 0.2% to 1.6% and a constant 0.1 M of choline chloride, the choline periodide solution was found to increase in absorbance and concentration. A straight line with R^2 = 0.9644 was thus obtained. Hence, by detecting the choline released from the enzymatic reaction and calculating its concentration, the butyric acid concentration could theoretically be identified and determined indirectly.

However, it was observed the absorbances of choline periodide of the samples that underwent enzyme hydrolysis (H) were lower than the absorbances of the samples that did not undergo hydrolysis (NH) at lower concentrations of butyric acid (0.2 - 1.0%), but the absorbances of choline periodide of H is higher than those of NH when the butyric acid concentrations increased to a certain level (1.2 - 1.6%) (Table 3). Theoretically, the choline periodide absorbances of the NH samples should not be higher than those of the H samples because there should be more choline present in

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Table 3. Butyric acid standard concentrations against choline periodide absorbance at 365 nm wavelength, measured using a microplate reader.

Well ID	Name	Well	Absorbance Unit (AU) at 365 nm	Repeat	Average Corrected ChoI AU
BLK	Tetrahydrofuran	A6	0.076	4	0.0775
		B6	0.079		
		C6	0.077		
		D6	0.078		
SPL1	0.2% ButyOH (H)	A1	0.222	4	0.1528
		B1	0.226		
		C1	0.244		
		D1	0.229		
SPL2	0.2% ButyOH (NH)	E1	0.232	4	0.1705
		F1	0.254		
		G1	0.252		
		H1	0.254		
SPL3	0.4% ButyOH (H)	A2	0.255	4	0.1808
		B2	0.252		
		C2	0.261		
		D2	0.265		
SPL4	0.4% ButyOH (NH)	E2	0.288	4	0.2138
		F2	0.294		
		G2	0.289		
		H2	0.294		
SPL5	0.6% ButyOH (H)	A3	0.265	4	0.1908
		B3	0.276		
		C3	0.260		
		D3	0.272		
SPL6	0.6% ButyOH (NH)	E3	0.281	4	0.2105
		F3	0.287		
		G3	0.298		
		H3	0.286		
SPL7	0.8% ButyOH (H)	A4	0.274	4	0.2063
		B4	0.283		
		C4	0.290		
		D4	0.288		
SPL8	0.8% ButyOH (NH)	E4	0.283	4	0.2090
		F4	0.286		
		G4	0.286		
		H4	0.291		
SPL9	1.0% ButyOH (H)	A10	0.280	4	0.2035
		B10	0.283		
		C10	0.280		
		D10	0.281		
SPL10	1.0% ButyOH (NH)	E10	0.291	4	0.2278
		F10	0.296		
		G10	0.300		
		H10	0.334		

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Table 3 (Cont.). Butyric acid standard concentrations against choline periodide absorbance at 365 nm wavelength, measured using a microplate reader.

Well ID	Name	Well	Absorbance Unit (AU) at 365 nm	Repeat	Average Corrected ChoI AU
SPL11	1.2% ButyOH (H)	A11	0.298	4	0.2385
		B11	0.315		
		C11	0.333		
		D11	0.318		
SPL12	1.2% ButyOH (NH)	E11	0.305	4	0.2265
		F11	0.299		
		G11	0.306		
		H11	0.306		
SPL13	1.4% ButyOH (H)	A8	0.315	4	0.2483
		B8	0.323		
		C8	0.332		
		D8	0.333		
SPL14	1.4% ButyOH (NH)	E8	0.300	4	0.2303
		F8	0.302		
		G8	0.307		
		H8	0.322		
SPL15	1.6% ButyOH (H)	A9	0.335	4	0.2670
		B9	0.338		
		C9	0.357		
		D9	0.348		
SPL16	1.6% ButyOH (NH)	E9	0.319	4	0.2465
		F9	0.324		
		G9	0.315		
		H9	0.338		

Table 4. Butyric acid concentrations against choline periodide absorbance ratio (H/NH) at 365 nm wavelength.

Sample	ButyOH Concentration (%)	ChoI AU Ratio (H/NH)
0.2% ButyOH	0.20	0.7044
0.4% ButyOH	0.40	0.8335
0.6% ButyOH	0.60	0.8797
0.8% ButyOH	0.80	0.9511
1.0% ButyOH	1.00	0.9385
1.2% ButyOH	1.20	1.0999
1.4% ButyOH	1.40	1.1448
1.6% ButyOH	1.60	1.2313
Average Sample Control AU (NH)	NA	0.2168

the H samples since these are the samples that have been subjected to hydrolysis by the enzyme. The NH samples were not subjected to hydrolysis by the enzyme hence the concentration of choline should be lower in these samples. At this point in time, it is not clear why such a phenomenon occurred. Thus, it is rather difficult to provide any meaningful insights for these observations.

After establishing the standard calibration curve to determine butyric acid concentrations using the potassium triiodide chemical method described, the butyric acid concentrations of the four vegetable samples previously extracted were determined. The average choline periodide absorbances (n = 4) at 365 nm for Kangkong, Kailan, Purple Kohlrabi Microgreen and *Cassia tora* (both hydrolysed and unhydrolyzed by BChE) measured are shown in Table 5. The corrected choline periodide absorbances were calculated based on the average choline periodide absorbance value of 0% Butyric acid (tetrahydrofuran only) obtained. The choline periodide hydrolysed (H) and unhydrolyzed (NH) ratios for the respective vegetable samples were then calculated after which the concentrations of butyric acid found in the four vegetable samples were then obtained based on the linear relationship established in the standard calibration curve (Figure 7), taking into consideration the dilution factor and the concentration of butyric acid spiked. The dilution factor was obtained by dividing the weight of the solid ester by the volume of its solvent (w/v %).

Hence, using this potassium triiodide chemical method, the concentration of butyric acid detected was 3.84% in Kangkong, 2.09% in Kailan, 7.43% in Purple

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Table 5. Choline periodide absorbances of the different vegetable extracts.

Well ID	Name	Well	Absorbance Unit (AU) at 365 nm	Repeat	Average ChoI AU	Corrected ChoI AU
BLK	Tetrahydrofuran	A6	0.076	4	0.0775	0.0000
		B6	0.079			
		C6	0.077			
		D6	0.078			
SPL1	*Cassia tora* (H)	A8	0.286	4	0.2808	0.2033
		B8	0.279			
		C8	0.277			
		D8	0.281			
SPL2	*Cassia tora* (NH)	E8	0.272	4	0.2778	0.2003
		F8	0.268			
		G8	0.279			
		H8	0.292			
SPL3	Kailan (H)	A7	0.254	4	0.2560	0.1785
		B7	0.259			
		C7	0.255			
		D7	0.256			
SPL4	Kailan (NH)	E7	0.295	4	0.2890	0.2115
		F7	0.290			
		G7	0.282			
		H7	0.289			
SPL5	Kangkong (H)	A9	0.248	4	0.2480	0.1705
		B9	0.254			
		C9	0.243			
		D9	0.247			
SPL6	Kangkong (NH)	E9	0.278	4	0.2778	0.2003
		F9	0.271			
		G9	0.280			
		H9	0.282			
SPL7	Purple (H)	A9	0.248	4	0.2345	0.1570
		B9	0.239			
		C9	0.228			
		D9	0.223			
SPL8	Purple (NH)	E9	0.241	4	0.2565	0.1790
		F9	0.261			
		G9	0.263			
		H9	0.261			

Table 6. Butyric acid concentrations of the different vegetable extracts calculated using the standard calibration curve of choline periodide absorbance ratio against butyric acid concentrations.

Sample	Sample Ester Weight (g)	ChoI Abs Ratio (H/NH)	Butyric Acid Concentration (%)	Corrected ButyOH Conc. (%)	Dilution Factor (Ester weight / 20 mL)	Final ButyOH Conc. (%)
Cassia tora	0.3370	1.0150	1.0202	0.5227	0.0168	31.02
Kailan	0.3361	0.8440	0.5326	0.0351	0.0168	2.09
Kangkong	0.2938	0.8514	0.5539	0.0563	0.0147	3.84
Purple Kohlrabi Microgreen	0.3486	0.8771	0.6270	0.1295	0.0174	7.43
ButyOH Spike	NA	NA	0.4975	NA	NA	NA

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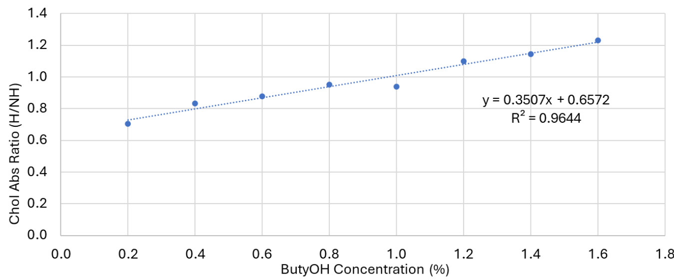

Figure 7. Standard calibration curve of choline periodide absorbance ratio against butyric acid concentration (%) using the potassium triiodide chemical method.

Kohlrabi Microgreen and 31.02% in *Cassia tora* (Table 6).

3.4 Validation studies

A summary of the results obtained for the validation of the potassium triiodide chemical method for the quantification of butyric acid is listed in Table 7.

3.4.1 Linearity

To verify the linearity of the potassium triiodide chemical method, analyses were carried out in triplicate for eight different concentrations of butyric acid (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6%). Results obtained for the ChoI absorbance ratio vs. butyric acid concentration were plotted and a regression analysis, expressed as determination coefficient (R^2), was performed.

Based on the analyses, the potassium triiodide chemical method showed a linear relationship in a butyric acid concentration range of 0.2 – 1.6% (Figure 7), with a coefficient of determination of $R^2 = 0.9644$.

3.4.2 Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) of the potassium triiodide chemical method were calculated using eight concentrations of butyric acid (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6%) in triplicate. The gradient (m), and standard deviation of intercept (sb_0) were determined from the ChoI absorbance ratio vs. butyric acid concentration graph. The following formulas were used to calculate the LOD and LOQ:

$$LOD = (3.3 \times sb_0)/m$$

$$LOQ = (10 \times sb_0)/m$$

Based on the range of butyric acid concentrations used, the LOD and LOQ of the potassium triiodide chemical method are 5.66% butyric acid and 17.15% butyric acid, respectively. These results represent the lowest amount of butyric acid which can be reliably detected and quantified.

Table 7. Validation parameters of the potassium triiodide chemical method.

Parameters	
Linearity (R^2)	0.9644
LOD (% butyric acid)	5.66
LOQ (% butyric acid)	17.15
Accuracy (%)	87.23 (*Cassia tora*) 91.44 (Kailan) 97.49 (Kangkong) 97.73 (Purple Kohlrabi Microgreen)

3.4.3 Accuracy study

The accuracy of the potassium triiodide chemical method was evaluated by comparing the butyric acid concentrations obtained using this chemical method with that of the HPLC method. Table 8 compares the concentrations of butyric acid obtained using the HPLC method and the potassium triiodide chemical method for the four vegetable samples. Results obtained from the quantification of butyrates found in *Cassia tora*, Kailan, Kangkong and Purple Kohlrabi Microgreen based on the chemical method were within 87.23%, 91.44%, 97.49% and 97.73% respectively when compared to the results obtained using HPLC.

Based on the results obtained, the butyric acid concentrations of 3 out of 4 of the vegetable extracts measured using the chemical method were above 91% when compared to the HPLC method. This shows that the potassium triiodide chemical method is comparable to the highly regarded HPLC method.

3.4.4 Precision study

The precision of the potassium triiodide chemical method was evaluated using the eight concentrations of butyric acid from the calibration curve (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6%) with n = 4 as well as based on the results obtained using the different vegetable samples, also with n = 4. Results are expressed as standard

Table 8. Determination of accuracy - comparison of the potassium triiodide chemical method and HPLC in the quantification of butyric acid.

Sample	Potassium Triiodide chemical method ButyOH Conc. (%)	HPLC Method ButyOH Conc. (%)	Accuracy (%) of chemical method
Cassia tora	31.02	35.56	87.23
Kailan	2.09	2.28	91.44
Kangkong	3.84	3.93	97.49
Purple Kohlrabi Microgreen	7.43	7.60	97.73

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Table 9. Results obtained for the various precision parameters for the potassium triiodide chemical method.

	Concentration of Butyric Acid	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6
		n = 4							
Enzyme hydrolysed (H)	Std Deviation	0.0096	0.0059	0.0071	0.0071	0.0014	0.0144	0.0085	0.0100
	Mean	0.2303	0.2583	0.2683	0.2838	0.2810	0.3160	0.3258	0.3445
	Relative Standard Deviation (%)	4.1714	2.2662	2.6601	2.5147	0.5033	4.5420	2.5973	2.9076
Non - enzyme hydrolysed (NH)	Std Deviation	0.0107	0.0032	0.0072	0.0033	0.0195	0.0034	0.0099	0.0100
	Mean	0.2480	0.2913	0.2880	0.2865	0.3053	0.3040	0.3078	0.3240
	Relative Standard Deviation (%)	4.3178	1.0992	2.4878	1.1576	6.3938	1.1074	3.2317	3.0967

	Vegetable extracts	*Cassia tora*	Kailan	Kangkong	Purple Kohlrabi
		n = 4			
Enzyme hydrolysed (H)	Std Deviation	0.0039	0.0022	0.0045	0.0112
	Mean	0.2808	0.2560	0.2480	0.2345
	Relative Standard Deviation (%)	1.3757	0.8438	1.8331	4.7804
Non - enzyme hydrolysed (NH)	Std Deviation	0.0105	0.0054	0.0048	0.0104
	Mean	0.2778	0.2890	0.2778	0.2565
	Relative Standard Deviation (%)	3.7918	1.8526	1.7235	4.0453

deviation, mean and RSD % in Table 9. It is noteworthy that the RSD % values obtained are all below 5% (except for non-enzyme hydrolysed 1.0% butyric acid).

4. Conclusion

The potassium triiodide chemical detection method developed in this study using potassium triiodide was found to be comparable to the HPLC method. This new chemical method is reliable when used to detect and quantify butyric acids and is very much cheaper as there is no need to acquire any specialised instruments which require regular maintenance thus adding to the cost lastly, there is no need for skilled professionals with specialized training to operate these instruments compared to the HPLC method.

Conflict of interest

The authors declare no conflict of interest.

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