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AN INNOVATIVE METHOD OF DISPERSIVE THREE LIQUID MICROEXTRACTION COMBINED WITH HPLC-UV FOR THE DETERMINATION OF VITAMIN B_1 IN SOUR CHERRY JUICE

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The present study examined an innovative method for determining the level of vitamin B_1 in sour cherry juice. Dispersive three liquid microextraction (DTLME) is a rapid, simple, and sensitive technique. Separation was accomplished using a C_{18} column. The optimum chromatographic conditions were found to be: a mobile phase of 5% methanol and 95% aqueous phase (1 M KH_2PO_4 water solution), a flow rate of 1.0 mL min^{-1} and a detection wavelength of 260 nm. The extraction efficiency of vitamin B_1 was influenced by factors such as sodium chloride, the type and volume of disperser, and the extraction solvents. The limits of detection (LOD) and quantification (LOQ) of the proposed approach were 0.15 and 0.5 ng mL^{-1}, respectively, for vitamin B_1. The relative standard deviation (RSD) for the three replicate determinations at 10 ng mL^{-1} was less than 3.63%. Appropriate linear behaviour over the observed concentration range was obtained with a value of R^2 >0.996 for vitamin B_1. This method was successfully applied to sour cherry juice samples. Sour cherry var. Gise (Prunus cerasus var. Gise), which was used in this research, is a local variety of sour cherry typically found in the high altitude areas of the Isfahan province in Iran.

Keywords: Dispersive three liquid microextraction (DTLME), High performance liquid chromatography-ultraviolet detector (HPLC-UV), *Prunus cerasus* var. Gise, Vitamin B_1, Thiochrome

INTRODUCTION

Vitamin B_1 is necessary for the synthesis of neurotransmitters such as gama-aminobutyric acid (GABA) and acetylcholine (Meador *et al.* 1993). It is a cofactor involved in amino acid catabolism and carbohydrate catabolism (Singleton and Martin 2001).

Sour cherries contain important compounds such as anthocyanins, catechins, phenolics, flavonal glycosides, melatonin, hydroxycinnamates, chlorogenic acid, cyanidins, and vitamins B and C (Burkhardt *et al.* 2001; Jacob *et al.* 2003). Scientists have found that the consumption of cherries can reduce muscle pain during running (Kuehl *et al.* 2010), apparently by preventing symptoms of muscle damage (Connolly, McHugh and Padilla-Zakour 2006), reducing muscle damage caused by intensive exercise (Bowtell *et al.* 2011), decreasing oxidative stress (Traustadottir *et al.* 2009), and protecting neuronal cells from cell-damaging oxidative stress (Kim *et al.* 2005). Cherry-derived compounds have also been demonstrated to have anti-gout (Blau 1950) and anti-inflammatory (Wang *et al.* 1999) effects.

One of the most important methods used to measure the level of vitamin B_1 present in a sample is to change it to the florescent thiochrome derivative; this method

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was first reported by Jansen in 1936 (Jansen 1936). Thiochrome is produced by the oxidation of vitamin B_1 in an alkaline medium with potassium hexacyanoferrate(III). Jansen's method was improved by Hennessy and Cerecedo in 1939 (Hennesy and Cerecedo 1939), who used a zeolite column and KCl, respectively, for the absorption and elution of vitamin B_1. In this method, as in Jansen's method, ferricyanide and alkaline conditions were used to change vitamin B_1 to thiochrome, but isobutanol was used for the extraction in the latter method. This procedure was further improved in 1940 by Najjar and Wood (Najjar and Wood 1940), who identified a factor increasing the yield. In 1965, Risinger and Pell found that the presence of methanol and ethanol in the oxidation medium could improve the production of thiochrome from vitamin B_1 (Risinger and Pell 1965). In the present study, we introduce a new method for measuring vitamin B_1 and for the extraction of thiochrome. This method had a high level of precision and accuracy.

A recent focus of research has been the development of analytical techniques that require smaller amounts of solvents and samples. Techniques such as solid phase microextraction (SPME) (Arthur and Pawliszyn 1990) and liquid phase microextraction (LPME) (Jeannot and Cantwell 1996, 1997) require small amounts of samples, but they have long extraction times (He and Lee 1997). To minimize the extraction time and the amount of samples and solvents required, Rezaea *et al.* developed dispersive liquid-liquid microextraction (DLLME) (Rezaee *et al.* 2006). The DLLME technique uses two solvents; an organic solvent that is insoluble in water (the extraction solvent) and a water-soluble solvent (the disperser solvent). The polarity of vitamin B_1 (thiamine) and thiamine phosphates is too high for DLLME, and their partition coefficients for DLLME were too low; therefore, a derivation step had been used to convert them to thiochrome. Derivatizing vitamin B_1 to thiochrome was performed by two methods: solid phase extraction and isobutanol extraction (Lawrance 2007). In the present study, isobutanol extraction method is used in order to convert vitamin B1 into thiochrome, but 3-methyl1-butanol is used instead of isobutanol.

In this procedure, the vitamin B_1 was combined with alkali and ferricyanide and was shaken to convert the vitamin B_1 into thiochrome. Then, the mixture was centrifuged to separate the layers, and the thiochrome was extracted with the DLLME method. However, in our new DLLME method three phases was formed and therefore, a new approach to separation was developed: dispersive three liquid microextraction (DTLME). In this technique, the efficiency of extraction depends on the type of disperser and the extraction solvent used.

EXPERIMENTAL

Chemicals and Reagents

Analytical grade reagents (99.8% acetic acid, 99.9% ethanol and chloroform), HPLC grade solvents (99.9% methanol and 99.9% acetonitrile), and ultrapure reagents (3-methyl1-butanol) were purchased from Merck (Darmstadt, Germany). Potassium hexacyanoferrate(III) (K_3FeCN_6, pure crystals), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). Authentic standard, 99.6% vitamin B_1 (thiamine hydrochloride) was purchased from Amin Pharmaceutical Co. (Isfahan, Iran). We produced de-ionised water using a Milli-Q (Merck, Darmstadt, Germany) reverse osmosis purification system. A preliminary standard of vitamin B_1 was formed by weighing out 1.00 mg of thiamine hydrochloride (vitamin B_1) into a 10 mL volume flask. By adding ultrapure water, the volume of standard was brought up to 10 mL.

Instrumentation

The HPLC system (Waters, Milford, MA, USA) included a Waters 2487 dual λ absorbance detector. The volume of the injection loop was 10.0 µL. Separation was performed on a HiQ Sil-octadecyl base (C18HS) column (25 cm × 4.6 mm with a 5 µm particle size) from Shinwa (Kyoto, Japan). The optimum chromatographic conditions were found to be: a mobile phase consisting of 5% methanol and 95% aqueous phase (1 M KH_2PO_4 water solution), a flow rate of 1.0 mL min^{-1}, a detection wavelength of 260 nm, and a mobile phase pH of 7.00. The isocratic elution method was used. For centrifugation, we used a Hettich centrifuge, model D-7200 (Tuttlingen, Germany). The pH meter was purchased from Metrohm (Herisau, Switzerland). The syringes (1.0 mL and 100 µL) were purchased from Hamilton Co. (Reno, NV, USA).

Plant Identification

Sour cherry var. Gise (*Prunus cerasus* var. Gise), which was used in this study, is a local variety of sour cherry with a large stone, double flowers, double fruits, dark red skin, and dark red juice. This variety is typically found in the high altitude areas of the Isfahan in Iran (Agricultural and Natural Resources Research Center of Isfahan).

Standard Solution and Calibration Curves

A stock solution (100 ng mL^{-1}) of vitamin B_1 was prepared by dissolving 1 mg of vitamin B_1 in 10 mL of de-ionised water in a calibrated flask. The temperature was adjusted to 4°C. We diluted the stock solution to make working solutions. The external standard method was used for the quantitative analysis. An appropriate amount of the stock solution was added to the sour cherry juice to prepare sour cherry juice standards. The calibration curve was achieved based on the concentration of vitamin B_1 and its corresponding peak area. The analyte concentration was calculated based on the calibration curve.

Preparation of Sour Cherry Juice

The sour cherry juice was first filtered with Whatmann filter paper no. 40. A volume of 10 mL of the filtered sour cherry juice was centrifuged for 10 minutes at 4,500 rpm. Then, the upper layer was collected using a syringe and filtered using a 0.22 µm syringe filter. The sour cherry juice was prepared daily.

DTLME procedure

For DTLME, 1.00 mL of the sour cherry juice, which was filtered with a 0.22 µm polytetrafluorethylene (PTFE) syringe filter, was poured into a 10 mL conical bottomed glass test tube, and then 2 mL of 3-methyl-1-butanol was added. The mixture was shaken for 10 seconds and then 2 mL of oxidant solution (a mixture of 1 mL of 0.06 M K_3FeCN_6 and 1 mL of 1 M NaOH) was added to the mixture. Then the solution was shaken for another 10 seconds. Two different phases were formed in the test tube: an organic phase and an aqueous phase; the two layers were then separated by centrifugation. We rapidly injected 0.5 mL methanol (disperser solvent) and 0.3 mL chloroform (extraction solvent) into the sample using a 1 mL Hamilton syringe. When the chloroform dispersed, a cloudy coloured solution was formed. At this stage, fine droplets of chloroform which had extracted the thiochrome, settled down at the bottom of the test tube. Then the sample was centrifuged for 10 minutes at 4,500 rpm. At this point three separate phases were formed in the test tube. The upper layer is an organic phase containing 3-methyl-1-

buthanol, the middle layer is an aqueous phase, and finally, the lowest layer is an organic layer that contains chloroform. The sedimented phase was then separated using a 1 mL Hamilton syringe and it was transferred into a small vial. The small vial was then placed under nitrogen flow. When the content dried up, it was dissolved in 0.2 mL of methanol and was injected into the HPLC system.

RESULTS

Adjusting the High Performance Liquid Chromatography-ultraviolet Detector (HPLC-UV) System

The maximum UV spectrum absorption of thiochrome was previously reported to be 260 nm (Chen and Wolf 2007), so the HPLC wavelength was adjusted to 260 nm. We used a C_{18} column and examined different concentrations of the aqueous phase (KH_2PO_4 water solution) and methanol. We found that when the mobile phase consisted of 5% methanol and 95% aqueous phase (1 M KH_2PO_4 water solution), it resulted in the sharpest peak and the best resolution for thiochrome. Figure 1(a) shows the HPLC chromatograms of vitamin B_1 for the standard solution after 3-metyl1-butanol extraction. The retention time of thiochrome was 3 minutes.

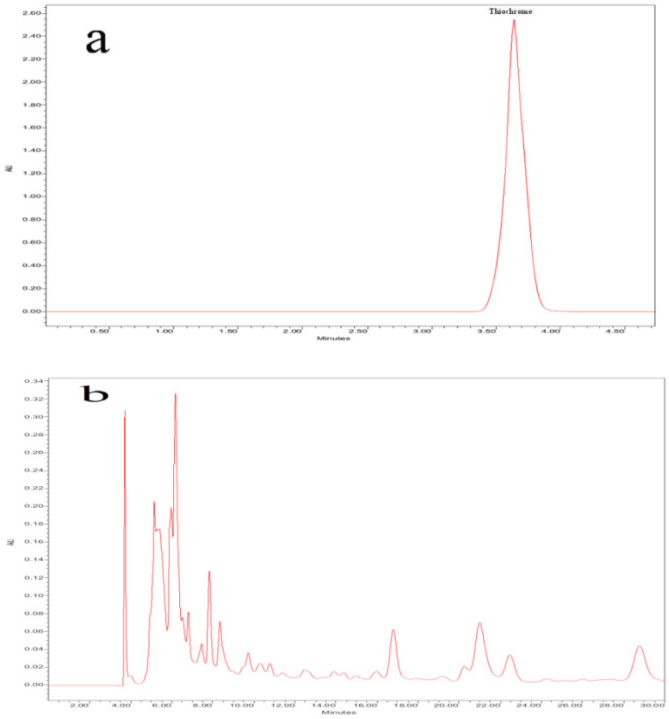

Fig. 1: Representative HPLC chromatograms of the standard solution of vitamin B_1: a) after 3-metyl1-butanol extraction; b) the sour cherry juice without extraction; c) the sour cherry juice after extraction using DTLME (*continued on next page*).

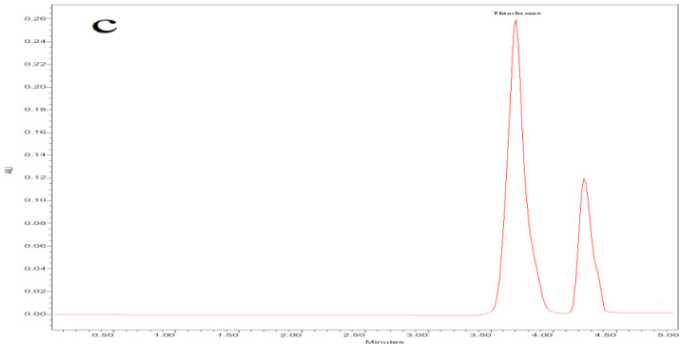

Fig. 1: (*continued*)

DTLME Method Optimisation

In this study, the type and volume of extraction and the disperser solvent, the volume of 3-methyl1-butanol, and the salt effect were studied and optimised.

Type of Disperser Solvent

The disperser solvent should be miscible in the extraction solvent and sample solution. To select the best disperser solvent for determining the concentration of vitamin B_1 present in sour cherries, we studied the effects of methanol, ethanol, and acetonitrile as disperser solvents. We found that methanol was the most appropriate solvent to use as a disperser because it produces the sharpest peaks with less interference (Fig. 2).

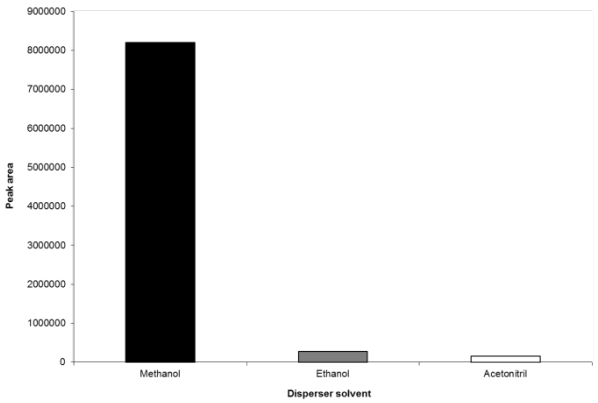

Fig. 2: The influence of the disperser solvent type on DTLME.

The Influence of the Disperser Solvent Volume on DTLME

To study the disperser solvent volume on the results of DTLME, the volume of extraction solvent was kept the same (chloroform, 0.3 mL), but the volumes of methanol were changed from 0.5 mL to 1.5 mL (0.5, 1.0, 1.5 mL). It was observed that the chromatogram peak areas were constant for these three volumes of methanol (Fig. 3).

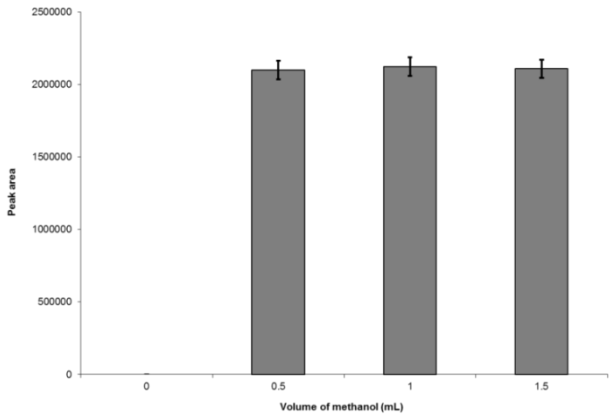

Fig. 3: The influences of the disperser solvent (methanol) volume on the results of the DTLME of vitamin B_1.

Type of Extraction Solvent

The extraction solvent should be immiscible in water, and its density must be higher than the density of water. In the present study, chloroform (density 1.48 ng mL^{-1}) was found to be appropriate to use as the extraction solvent.

The Comparative Influence of the Extraction Solvent Volume on DTLME

To examine the effects of the extraction solvent volume on DTLME, the volume of disperser solvent was kept the same (methanol, 0.5 mL), but the volume of chloroform was varied from 0.1 mL to 0.3 mL (0.1, 0.2, 0.3 mL). It was observed that the chromatogram peak areas increased when the chloroform volume increased (Fig. 4).

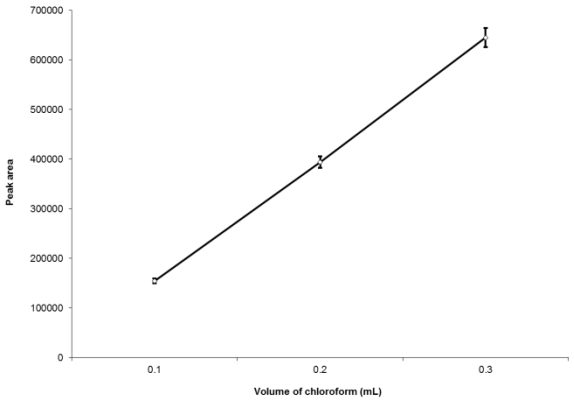

Fig. 4: The influence of the extraction solvent (chloroform) volume on the extraction efficiency.

The Comparative Influence of the 3-methyl1-butanol Volume on DTLME

To study the effects of different volumes of 3-methyl1-butanol on the DTLME, different volumes of 3-methyl-1-butanol (0.1, 0.2, 0.3, 0.4 mL) and 0.5 mL of methanol (disperser solvent) and 0.3 mL of chloroform (extraction solvent) were added to the sample. It was observed that an increase in the volume of 3-methyl1-butanol caused an increase in the peak area, but the increase up to 0.2 mL caused some interference in the chromatogram peaks, which, in turn, caused a decrease in the efficiency of the extraction (Fig. 5).

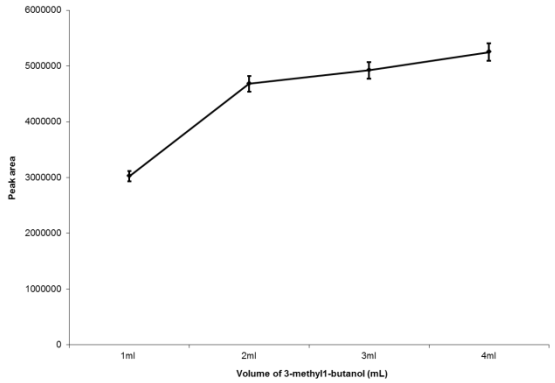

Fig. 5: The influence of the 3-methyl1-butanol volume on the results of DTLME.

The Influence of Sodium Chloride on DTLME

To study the effects of sodium chloride on DTLME, we added different concentrations of NaCl from 0–0.07 M to the mixture. The chromatogram peak areas were increased with the increase in the NaCl concentration, but salt caused some intrusive peaks in the chromatogram (Fig. 6); thus, we did not add any NaCl under the optimum condition.

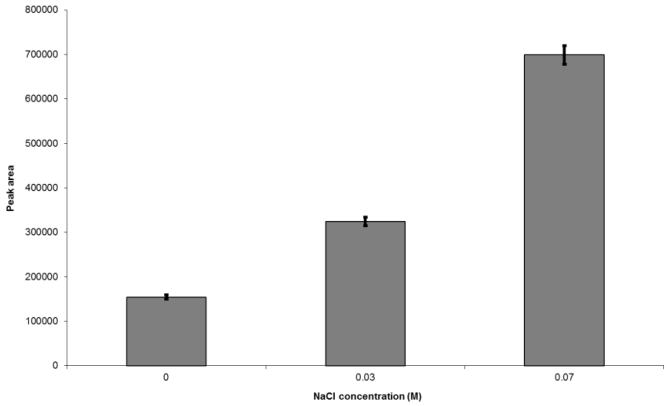

Fig. 6: The influence of sodium chloride on the results of DTLME.

DISCUSSION

Recovery Testing

The recovery rates of the samples, with six different concentrations extracted with the DTLME method, are shown in Table 1. The average recovery (%) was 103.24.

Table 1: The analytical results for vitamin B_1 in sour cherry juice samples (mean ± SD, n = 6).

Analyte	Amount added	Amount found (ng mL^{-1})	Recovery (%)
Vitamin B_1	0	0	–
	10	11.39±1.59	113.9%
	30	29.73±0.26	99.1%
	50	53.48±3.72	106.9%
	70	67.44±2.47	96.3%
	100	99.99±0.01	100.0%

Method Validation

The linearity of the method and its sensitivity are described in Table 2. The limit of detection (LOD; LOD = 3.3σ/S) and the limit of quantification (LOQ; LOQ = 10σ/S) were calculated (Samanidou, Nika and Papadoyannis 2007), where the standard variation was σ (n = 6). The relative standard deviations (RSDs) were obtained for the method repeatability and reproducibility. The RSD for the method repeatability was obtained with determinations of sour cherry juice samples for three replicates and six concentrations of the analyte (0, 0.01, 0.03, 0.05, 0.07, 0.1 μg mL^{-1}) on the same day. The RSD for the method reproducibility was obtained via the determination of the analyte concentrations in sour cherry juice samples prepared as three replicates at six levels of analyte (0, 0.01, 0.03, 0.05, 0.07, 0.1 μg mL^{-1}) on three days and three times each day. Typical chromatograms of blank sour cherry juice and spiked sour cherry juice samples (after DTLME) are shown in Figure 1.

Table 2: The analytical performance of HPLC-UV for vitamin B_1 on the C18 column.

Analyte	Calibration curve	R^2	Linear range (μg mL^{-1})	RSD(%) repeatability	RSD(%) reproducibility	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Vitamin B_1	y = 152914x + 311998	0.996	0.003–0.140	3.63	3.92	0.15	0.50

Application

Various analytical methods have been developed for the extraction and determination of vitamin B1, including liquid chromatography with mass spectroscopy (Chen and Wolf 2007), HPLC with ultraviolet detection (Agostini-Costa *et al.* 2007, Chen *et al.* 2011, Patil and Srivastava 2013), and liquid chromatography with diode-array detection (Chen and Wolf 2007; Jin *et al.* 2012), among other techniques. The DTLME method is simple, rapid, and sensitive and has high accuracy. The DTLME method overcomes some of the disadvantages of the other methods, such as the matrix effect, difficult operation, time-consuming steps, low sensitivity, toxicity, and the need for expensive solvents.

Although the concentration of vitamin B_1 was determined HPLC in other studies, the present research allowed for the extraction and detection of vitamin B_1 using low amounts of sample and reagents by utilising an innovative DTLME method. A comparison of the limit of detection of vitamin B_1 using HPLC in different studies is presented in Table 3.

Table 3: Comparison of detection limits of vitamin B_1 using HPLC in different studies.

Method	Sample	LOD	Reference
HPLC-UV	Tarhana (a traditional food)	0.5 mg L^{-1}	Ekinci and Kadakal 2005
RP HPLC-UV	Multivitamin tablets	0.6250 µg mL^{-1}	Amidzic *et al.* 2005
HPLC-fluorescence	Cooked sausages	0.15 mg 100 g^{-1}	Valls *et al.* 1999
HPLC-UV	Enriched flavored milk mixes	0.04 µg mL^{-1}	Agostini-Costa *et al.* 2007
DTLME-HPLC-UV	Sour cherry juice	0.155 ng mL^{-1}	Present work

Determining the concentration of vitamin B_1 in sour cherry juice samples was the aim of the present study. The results on actual samples are shown in Table 1. For the spiked sour cherry juice, samples had a recovery range of 96.3%–113.9%. The concentration of vitamin B_1 in the sour cherry juice samples was 69.1±3.5 ng mL^{-1}. To examine the validity of the method, 70 ng mL^{-1} of vitamin B_1 was spiked into sour cherry juice samples, and the concentration obtained was found to be 141.6±7.2 ng mL^{-1}. This value is consistent with the sum of vitamin B_1 naturally present in the sour cherry juice and the 70 ng mL^{-1} of the target vitamin added to the sample. Therefore, the obtained data showed satisfactory recovery. Representative chromatograms of sour cherry juice after extraction using DTLME are shown in Fig. 1(c). This cleanup method worked well and produced sharp thiochrome peaks with less interference peaks. The 3-methyl-1-butanol cleanup was easier to perform and perhaps more reproducible, than the isobutanol technique, but either technique can be used for DTLME.

CONCLUSION

In this study, an innovative DTLME-HPLC-UV method for the determination of vitamin B_1 in sour cherry juice was evaluated. The extraction efficiency of vitamin B_1 was influenced by factors such as sodium chloride effect, the volume of 3-methyl-1-butanol, and the type and volume of disperser and extraction solvents. The method showed advantages compared with other conventional methods such as better clean up (sharper thiochrome peaks with less interference) and a shorter extraction time. It also produced sharper thiochrome peaks with less interference. The relative recovery of vitamin B_1 in sour cherry juice ranged from 96.3%–113.9%. The DTLME method was rapid, simple, and reproducible, with high linearity.

Conflicts of Interest

None of the authors has any conflict of interest to declare.

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