DETERMINATION OF CYCLAMATE CONTENT IN SOME FOOD PRODUCTS USING UPLC-MS/MS

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Abstract. Additives used in food pose a risk to consumers' health, and cyclamate is one of the chemical sugars. In this study, cyclamate content in 38 food samples including beverage, cake, candy, milk, and juice was analyzed using ultra-performance liquid chromatography, tandem mass spectrometry (UPLC-MS/MS). The analysis conditions were: the liquid chromatography column UPLC BEH C18 2.1 × 100 mm 1.7 μm, waters, USA; mobile phase: MeOH:H_2O (50:50) adding 0.1 % HCOOH, deionized water. The ultimate test sample which passed 0.22 μm PTFE membrane filter results in retention time of 4.05 minutes, and flow rate of 0.2 mL/min. The calibration curve for cyclamate was linear in the range of 5 - 100 mg/L with $R^2 = 0.9955$. The limit of detection (LOD) and limit of quantification (LOQ) was 2.92 and 9.72 mg/kg, respectively. The average recoveries of the whole analytical procedure ranged from 83.38 to 93.40 %. The bias and RSD of the method was 0.015 and 0.17 %, respectively. The fragment ions of 79.84 and 95.79 m/z have been measured and used for quantitative research and confirmability, respectively. The results showed that 23/38 food product samples from markets and supermarkets in Ha Noi city contained cyclamate, accounting for 60.53 %, with concentrations ranging from 10.9 to 178.1 mg/kg. However, the content of cyclamate in all samples have met the standards as regulated in Circular No. 08/2015/TT-BYT. In addition, cake and candy samples used cyclamate more frequently compared to other analyzed food types.

Keywords: artificial sweetener, cyclamate, food safety, food addictive, UPLC-MS/MS.

Classification numbers: 3.2.1, 3.2.3.

1. INTRODUCTION

Cyclamate is often in sodium cyclamate and calcium cyclamate forms. Cyclamate is used like any zero-calorie sweetener that is 30 time sweeter than that of sucrose (sugar cane). Cyclamate is not harmful to teeth, is suitable for the diabetics, and it is one of the cheapest sweeteners. Cyclamate is heat stable and has a long shelf life; it is easy to storage, has no water

absorption from the air and no fermentation. Therefore, it is used as supplements in food industry, and medical industry [1, 2, 3].

Currently, the Food Standards Commission (CODEX) takes the cyclamate into the list of additives authorized for use in food for certain food groups [4] and according to International Agency For Research on Cancer (IARC), cyclamate is placed in Group 3, which is not classified as a human carcinogenic group.

In Viet Nam, cyclamate has been banned for use in food. However in 2013, Viet Nam Food Administration issued Circular 27/2012/TT-BYT- A Guide Food Additives Management (amended in Circular No. 08/2015/TT-BYT; edit and additional provisions of Circular 27/2012/TT-BYT on 30/11/2012 – A guide to the management of food additives), which allows the use of cyclamate as food additive, but has specified the threshold limit for each type of different food. So far, no research has confirmed the cause of carcinogenic for human of cyclamate, however, regardless of what type of chemical sugar, anything used beyond the allowable threshold will be harmful to human health [5].

There have been number of studies on cyclamate detection and quantitative for different samples [3, 6, 7]. Of which, liquid chromatography techniques with UV detector (LC-UV) is one of common methods. Although the LC-UV is the standard method (according to Europe) [8], it often causes a possitive error, in particular for the food samples with complex compositions.

Some other researchers also use the LC/MS/MS, however, this technique has some drawbacks such as not able to research the fragmentation mechanism, the differences between the isomers which further identifies the chemical structure, due to the soft ionization technique, pseudo molecular ion ($[M-H]^-$ in negative mode or $[M+H]^+$ in positive mode) is observed instead of molecular ion. The HPLC-MS/MS technique has some advantages including a higher sensitivity, accuracy, and capability to detect substances in low concentrations (ng/mL). Therefore, the HPLC-MS/MS technique has been widely used for many recent years.

Sheridan *et al*. developed the cyclamate identification method in some food samples using the HPLC-MS/MS technique [6]. Samples were extracted with 10 mL of deionized water, cyclamate was separated on the C18 column with 0.15 % acetic acid, the calibration curve has a linear correlation coefficient $R^2 > 0.9998$, a good application method for determining the cyclamate with the detection limit of 0.05 μg/g. In 2014, Shah *et al*. also developed and verified the determination of cyclamate using the HPLC-MS/MS with the internal standard cyclamate-D11. The sample was also extracted with deion water, the recovery fluctuates between 72 and 110 % with a relative standard deviation of 3 to 15 % [7].

In the study of Janvier *et al.* [9], UPLC-MS/MS was used to analyze cyclamate and other sweeteners in beverage and food supplements. It was found that only cyclamate in one beverage exceeded the maximum level with 13 %, all other samples measured in food were around or below the maximum level. However, in food supplements, the cyclamate was found in 40/52 samples and the maximum exceeded 200 % of the maximum level.

In Viet Nam, there have not been any publication on research to detect cyclamate by HPLC-MS/MS. With the aim towards being a harmony in the methods of analysis with other countries when participating in World Trade Organization, this article introduces the method of detecting the cyclamate quantitative in some food samples using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). This method has a high sensitivity and selectivity, and simple sample extraction procedure. The analysis using the common column type (UPLC BEH C18 2.1 × 100 mm 1.7 μm, waters, USA), simple operation and requires less analysis time. In addition, it is easily applied in the facilities with HPLC system. The sample is

extracted with a mixture of solution MeOH: H_2O (1:9 v/v) then quantitatively analyzed using UPLC-MS/MS. The method has been determined and verified on the conformity of the chromatography system, characteristics, accuracy, linearity range, limit of detection, limit of quantitative which all meet requirements according to European Community regulations [10].

2. MATERIALS AND METHODS

2.1. Reagents, chemicals and samples

Methanol (MeOH) was purchased from Merck, Germany, deion water used for HPLC (Merck, Germany). Formic acid was analytical grade (Spain). Standard cyclamate (99.8 %) from USA. A stock standard solution (100 mg/L) was prepared in methanol, conserved at -20 °C and used within 3 months. From stock solution, working solutions of 5.0; 10; 15; 20; 25; 50 and 100 mg/L using MeOH: H_2O (1:9 v/v). Mobile phase solvent A is methanol; mobile phase solvent B is deionized water with 0.1 % formic acid.

Total 38 samples were taken from the local market, and supermarket in Ha Noi. Of which, there are 15 beverage samples, 7 cake samples, 5 candy samples, 5 milk samples and 6 juice samples. All samples were found to contain cyclamate diluted into this range for quantitation.

2.2. Chromatography conditions

The UPLC-MS/MS (Waters, USA) with a Waters Acquity TQ Detector and Masslynx 4.1 software. Separation was carried out on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm). The mobile phase started with 10 % solvent A, held for 1 minute then increased to 100 % solvent A and held for 2 minutes. Total running time was 10 minutes with the flow rate of 0.2 mL/min, and the injection volume of 10 μl. Multiple reaction monitoring (MRM) mode was applied to detect cyclamate with electrospray source operated in the negative ionization mode. The following parameters were optimal: in source temperature, 150 °C; desolvent temperature: 250 °C; desolvation gas flow rate, 500 L/hr. The MS capillary voltage, 3.0 kV; Cone voltage: 43 V; and vacuum pressure was 3.60 mBar. The retention time of cyclamate found 4.05 minutes (Figure 1). Other parameters are shown in Table 1.

2.3. Sample preparation

For solid samples: Weigh 1.0 g of mixed sample into a 50 mL polypropylene centrifuge tube and add in 10 mL MeOH: H_2O (1:9 v/v). The sample solution was then vortexed for 5 minutes for homogenized sample and vortexed for 10 minutes (5000 rpm) at room temperature. The supernatant was separated then the extraction procedures were repeated once. Take all supernatant from 2 extractions and centrifuged at 5000 rpm for 10 minutes at room temperature. The final supernatant was collected into a 20 mL volumetric flask then diluted to the volume with mobile phase; 1 mL of the solution was filtered with 0.22 μm PTFE membrane filter, desolvent and transferred to an autosampler vial, degassed and injected into UPLC-MS/MS.

For liquid samples: Dilute 1.0 mL sample in 9 mL MeOH:H_2O (1:9 v/v). The sample solution was then vortexed for 5 minutes. Took 1 mL of the supernatant and filtered with 0.22 μm PTFE membrane filter, desolvent and transferred to an autosampler vial, degassed and injected into UPLC-MS/MS.

3. RESULTS AND DISCUSSION

3.1. Optimal UPLC-MS/MS conditions

UPLC-MS/MS technique always has a high specification, the presence of the analyte is definitely confirmed through retention time, parent ion and product ions. In addition, according to the European Commission Decision 657/2002/EC [8], the specification is rated through the identification points (IP) and the ratio of confirmed and quantitative ions. A minimum of four points (IPs) was required, where each parent ion is counted as 1 point, each product ion is counted 1.5 points. For MS/MS techniques, precusor ions can form a wide variety of different product ions, depending on the technique of negative ionization or a positive ionization.

Positive ionization mode usually produces parent ion $[M+H]^+$, $[M+CH_3OH]^+$, $[M+Na]^+$... The cyclamate is an anion so the ionization in the negative ion mode will have higher sensitivity than the positive ionization mode. In this study, bombarding molecular ion was carried out in the negative ionization mode by preparing the standard solution concentration of 100 mg/L, flow rate of 0.2 mL/min, and performing direct pump mode (Table 1). The results were similar to that of Shah *et al.* [7] reported in 2014, which also found 1 parent ion and 2 fragment ions (178.03 > 79.84 and 178.03 > 95.79 m/z). Compared to the result of Sheridan *et al.* announced in 2008, method applied in our study was more specific, and selective because in the study of R. Sheridan *et al.* when implementing the LC-MS/MS technique, only a characteristic ion of 178 > 80 was found [6].
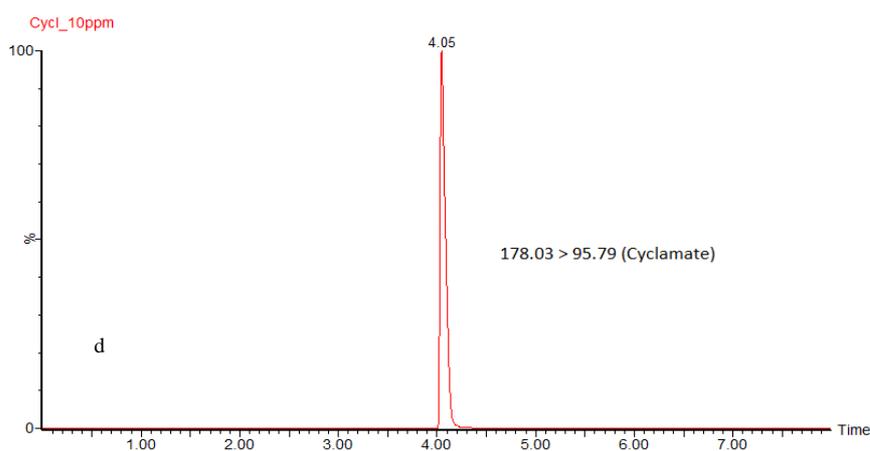

Figure 1. Chromatogram of cyclamate in standard solution (10 mg/L).

Table 1. Ion fragment energy of cyclamate.

Compound	Parention (m/z)	Productions (m/z)	Dwell time (s)	Cone voltage (V)	Collision Energy (eV)
Cyclamate	178.03	79.84[a]	0.161	34	26
(Retention time: 4.05 min)		95.79	0.161	34	16

Note: [a]*product ion (fragment) for quantification*

Compared to the techniques that many authors still perform during the cyclamate analysis, the LC-MS/MS technique has better performance. LC-MS/MS also has been used simultaneously to save analysis time, produce spectral fragments of molecular ions, the fragment ions, therefore the results guaranteed higher selectivity.

Table 2. Results of the limit of detection and quantitation of the method.

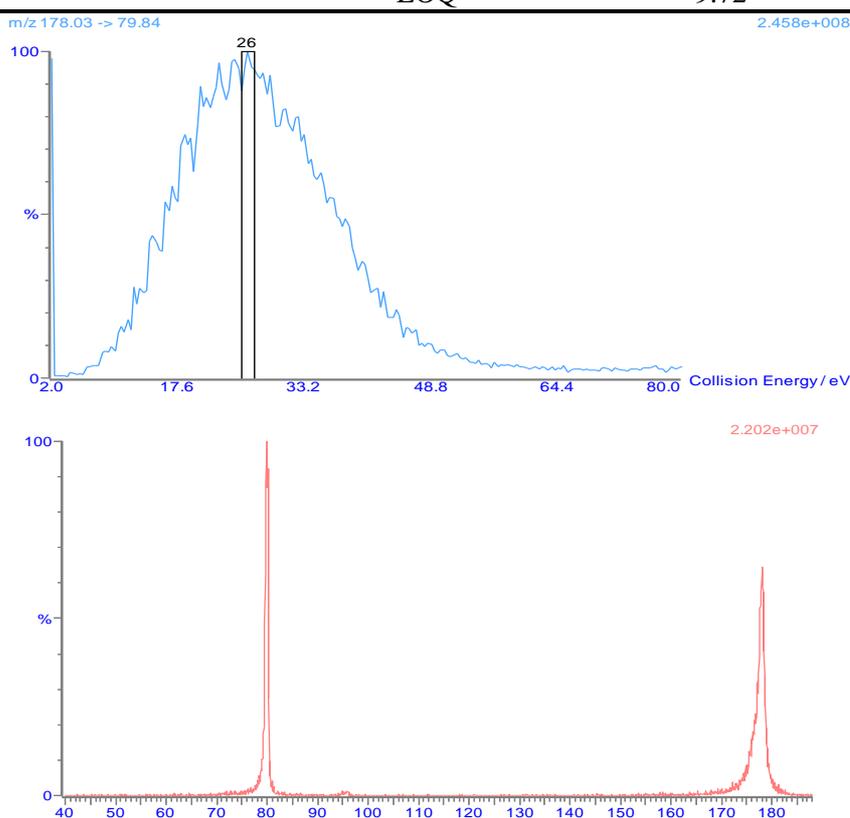| No.	Sample	Result (mg/kg)
(1)	S20_1	20.16
(2)	S20_2	18.62
(3)	S20_3	19.12
(4)	S20_4	21.00
(5)	S20_5	18.42
(6)	S20_6	20.22
(7)	S20_7	19.30
(8)	S20_8	18.56
(9)	S20_9	19.25
(10)	S20_10	18.06
	Average, X_{TB}	19.27
	SD	0.93
	LOD	2.92
	LOQ	9.72

Figure 2. Collision energy optimization (m/z 178.03 → 79.84).

In the study of Ordoñez *et al.* using LC-MS/MS, LODs identified ranged 0.05 - 10 mg/L after 50-fold dilution [11]. However, when developed and validated LC-MS/MS methods to determine cyclamate in a variety of food matrices, Shah R *et al.* found LODs and LOQs to be very low in comparison to this study (LODs: 0.1 and 0.6 mg/L for pomegranate juice and dried fig, respectively, LOQs were 0.3 and 1.6 mg/L). These limits were significantly lower than needed to analyse cyclamate when used as a food additive. However, the LODs was much higher (in the range of 1-20 mg/kg) and linearity range found up to 1300 and 67 mg/kg for cyclamic acid in foods and beverages using HPLC-UV, respectively [7].
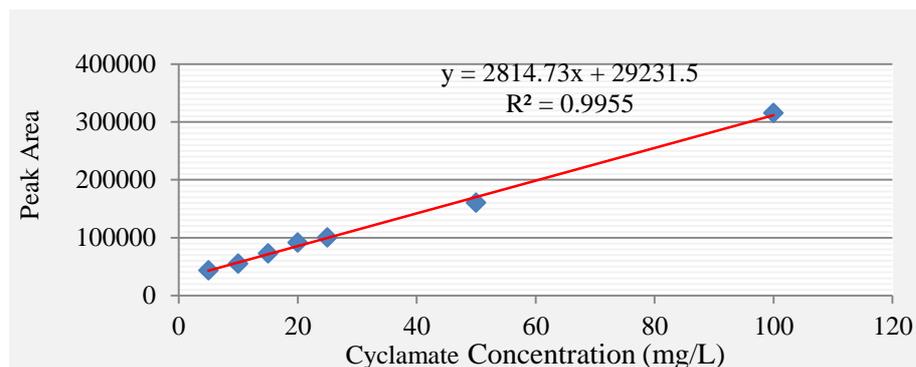

Figure 3. Calibration curve for cyclamate quantitative analysis.

3.3. The recovery

The recovery was measured on the standard addition samples at 3 concentrations 15; 30 and 60 mg/kg using calibration curve for calculations. At each concentration, the extraction independently carried out with a 5 time-replication. The result showed that the cyclamate recovery performance was quite high, averages ranged from 83.38 to 93.40 % with relative standard deviations (RSD) fluctuating between 0.07 and 0.17 % (Table 3).

Table 3. Results of the recovery (H%) and the relative standard deviation (RSD%).

Content (mg/kg)	Items	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average	SD	RSD (%)
15	C (mg/kg)	13.4	15	12.5	13.9	15.3	14.02	1.15	0.08
	H (%)	**89.4**	**99.9**	**83.4**	**92.5**	**101**	**93.40**		
30	C (mg/kg)	30	29.7	23.6	23.5	19.9	25.34	4.38	0.17
	H (%)	**100**	**99.0**	**78.7**	**78.2**	**76.2**	**84.44**		
60	C (mg/kg)	45.9	48.6	48.7	54.5	52.4	50.02	3.41	0.07
	H (%)	**76.5**	**81.0**	**81.2**	**90.8**	**87.4**	**83.38**		

3.4. Application of developed method to determine cyclamate in food product samples

The analytical results showed that 23/38 food products samples from markets and supermarkets in Ha Noi city contain cyclamate, accounting for 60.53 % of total samples taken in Ha Noi city with a concentration ranging from 10.9 to 178.1 mg/kg. Of which, the detected cyclamate content in candy samples range from 62.0 to 172.5 mg/kg, accounting for 80 % detected cyclamate of total candy samples tested. The cyclamate detection and content in some tested food samples are shown in Figures 4 and 5.
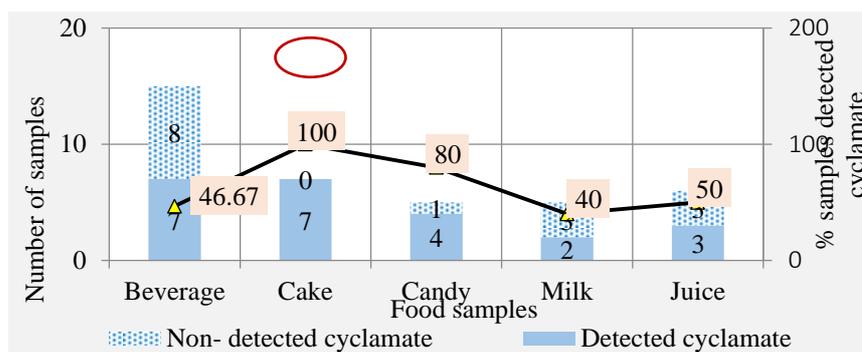

Figure 4. The results of cyclamate detection in tested food samples.

However, compare to the cyclamate content allowed in food products which follows the Circular No. 08/2015/TT-BYT issued by the Ministry of Health on 11/05/2015 [5], in beverage, milk, ice cream (250 mg/kg), cake (1600 mg/kg) and candy (500 mg/kg), all 38 tested samples have lower cyclamate contents than the permitted maximum level. The permitted maximum level in beverage, milk, and ice cream is similar to EU and UK standards for Brewed soft drinks [7, 12, 13], however, in Australia and New Zealand a much higher level is allowed (400 mg/kg) [14].
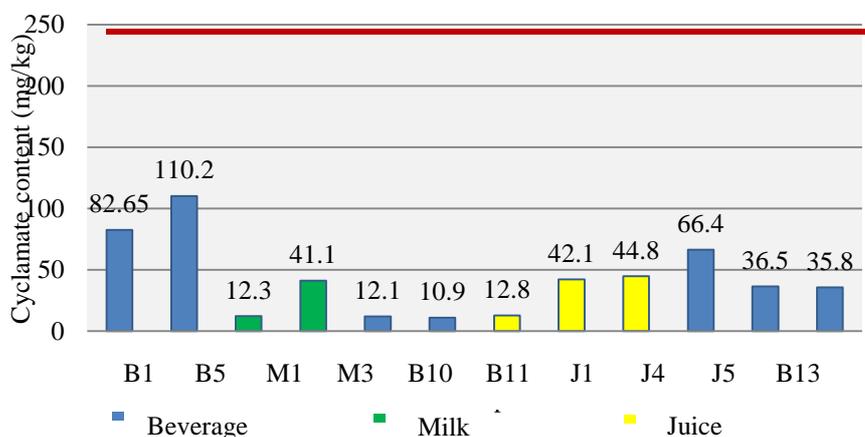

Figure 5. Cyclamate content detected in some beverage, milk, and juice samples.

4. CONCLUSIONS

This study developed the procedures for determining cyclamate in a number of food products by UPLC-MS/MS with high sensitivity and selectivity, simple extraction process, quick analysis time. Results identified the LOD and LOQ of 2.92 and 9.72 mg/kg, respectively. The bias is 0.015 % and recovery are in the range of 83.38 - 93.40 %. The IPs have been verified according to the European Community Decision 657/2002/EC. The procedure has been applied to the analysis of 38 food samples. The findings showed that 23/38 food samples containing cyclamate in the range of 10.9 - 178.1 mg/kg. Fortunately, the cyclamate content in all samples was still lower than that in permitted standard according to Viet Nam (Circular No. 08/2015/TT-BYT) and some other countries' regulations.

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