論　　文

食品添加物ブドウ果皮抽出物の含有成分のクロマトグラフィーによる評価

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キーワード：ブドウ果皮抽出物、高速液体クロマトグラフィー、アントシアニン、プロアントシアニジン、タンニン

概　要

既存添加物名簿に収載されている製造用剤ブドウ果皮抽出物について、HPLCによる含有成分の検討を行った。エキス中の成分をカラムクロマトグラフィーにより分離し、分光分析法に基づき12種の化合物（tryptamine, syringic acid, vanillic acid, ethyl gallate, (+)-catechin, (−)-epicatechin, luteoliflavan, quercetin, quercetin 3-*O*-glucuronide, myricetin 3-*O*-glucoside, procyanidin B-1, procyanidin B-2）を単離、同定した。またHPLCにより、主アントシアニンとしてmalvidin 3-*O*-glucosideを認めた。一方、大きなブロードピークについては、proanthocyanidin Bタイプで構成される縮合型タンニンオリゴマー画分であることが示唆され、ゲル浸透クロマトグラフィーにより平均分子量を求めた結果、数平均分子量は5999.6、重量平均分子量は21287.7であった。ブドウ果皮抽出物製品中のプロアントシアニジン含量を、バニリン・硫酸を加えて呈色させる方法によりcatechin換算で2製品を定量分析した結果、約60%の含有率が算出され、測定可能であることが示唆された。

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Development of HILIC-LC/MS method for direct quantitation of 2-acetyl-4-tetrahydroxybutylimidazole in caramel III with the qNMR certified standard

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Abstract

A method LC/MS with HILIC column (HILIC-LC/MS) was developed for direct quantitation of 2-acetyl-4-tetrahydroxybutylimidazole (THI), an undesired polar byproduct in caramel III colorants. To verify the reliability of the proposed analytical method for the quantification of THI in caramel III commercial products, we determined the absolute purity of a THI analytical standard using quantitative NMR (qNMR) and then performed absolute calibration and standard addition procedures using the analytical standard. The correlation coefficients were >0.99 and >0.97 for the absolute calibration and standard addition procedures, indicating satisfactory linearity of the respective calibration curves. The procedures also returned identical quantitation values in a sample. The THI content in six samples of caramel III commercial products in Japan was determined using the HILIC-LC/MS method. The THI content in each of these samples was lower than officially stipulated limits. The current JECFA standard method for determination of THI in caramel III by HPLC/UV using a 10-μm particle size C8 column with derivatization of THI-2,4-dinitrophenylhydrazone gave lower THI values than the proposed HILIC-LC/MS method due to sub-optimal peak separation by the column recommended in the JECFA standard method. Our data suggest that the analytical conditions of the current JECFA standard method should be improved.

Keywords : HILIC, LC/MS, qNMR, caramel, 2-acetyl-4-tetrahydroxybutylimidazole

I Introduction

Caramel III (caramel class III), ammonia caramel, is a widely used coloring additive in foods and beverages. Caramel III may contain small amounts of the undesired byproduct 2-acetyl-4-tetrahydroxybutylimidazole (THI). Because THI is reportedly immunotoxic,[1-3] limits of THI content in caramel III and analytical methods for its determination have been specified by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the European Union (EU), the Food Chemicals Codex (FCC), and in Japanese regulations for food additives.[4-7] All current THI standard analytical methods are based on the method established by Kröplien et al.[8]

The standard analytical method recommended by the JECFA involves purification of THI from caramel III via cation exchange chromatography using two different resins, derivatization of THI with 2,4-dinitrophenylhydrazine (DNPH) to its hydrazone (THI-DNPH), and finally detection of THI-DNPH using HPLC/UV (Fig. 1).[6] Determination of THI in caramel III through DNPH derivatization has the significant advantage of requiring only classical HPLC equipped with a UV detector. However, the JECFA standard method defines for the use of a 10-μm particle size C8 HPLC column, which is currently not commercially available and generally provides poorer separation capacity than 5-μm particle size C8 columns. This leads to unsatisfactory separation of THI-DNPH from other interfering substances and a consequent reduction in the reliability of quantitation values. In a previous study, we therefore attempted to establish a more reliable method for analyzing THI-DNPH using a

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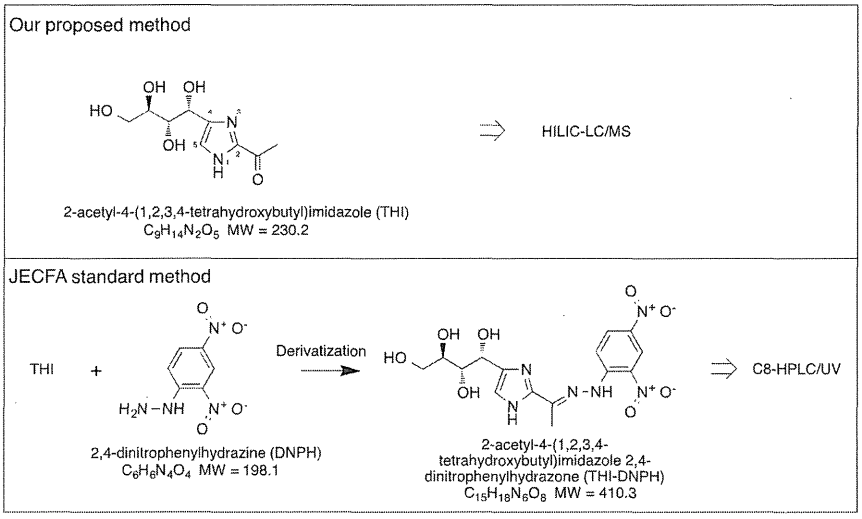

Fig. 1. Structures of 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) and its derivative, 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole 2,4-dinitrophenylhydrazone (THI-DNPH)

commercially available 5-μm particle size C8 column.[9] We investigated the effects of the mobile phase and reported that separation of the THI-DNPH peak is improved when 0.1 mol/L phosphoric acid/methanol (70:30) is used as the mobile phase. Compared with the JECFA standard method, the revised method indicated higher levels of THI in a caramel III commercial product. In order to verify the accuracy of the quantitative values obtained using the revised method, THI-DNPH in a product sample was also analyzed by LC/MS. The value obtained in the LC/MS analysis was equivalent to that obtained using the revised method. These results demonstrated the superiority of the revised method compared with the JECFA standard method. However, obtaining a true value for the THI content of a sample remains questionable due to several factors. First, no absolutely pure THI analytical standard is currently available on the reagent market. In addition, methods involving DNPH derivatization are based on the assumption that the THI-DNPH reaction yield from the THI analytical standard is identical to that from trace levels of THI as a byproduct in caramel III commercial products.

Several analytical methods for the determination of THI and other imidazole derivatives in foods or caramel III products have recently been published, including methods for the rapid and high-sensitivity analysis of THI and the imidazole derivative 2-methylimidazole (2-MI) using LC/MS[10], simultaneous analysis of THI, 2-MI, 4-methylimidazole (4-MI), and 5-hydroxymethylfurfural (HMF) in caramel colors and beverages using LC/MS/MS[11, 12], solid-phase extraction (SPE)-LC/MS[13], and SPE-UHPLC/MS/MS.[14] Hydrophilic interaction chromatography (HILIC) effectively separates and retains polar analytes, even though reverse-phase chromatography with C18 column (C18RPC) is generally inappropriate for the analysis of polar analytes due to the basic principles of the separation.[15] The feature of HILIC has been incorporated into a method for the simultaneous analysis of THI and 4-MI in coffee using supercritical fluid extraction (SFE)-LC/MS.[16]

A quantitative nuclear magnetic resonance (quantitative NMR: qNMR) technique has also been developed and is considered to be a primary ratio method.[17, 18] In qNMR, the purity or content of an analyte can be determined based on the ratio of the integral values of the specific signal of the analyte to that of an internal standard (IS), because the integral values of the analyte and IS are directly proportional to the number of protons per resonance line multiplied by the molar concentration of the analyte and the IS. Therefore, when a certified reference material (CRM) is used as the IS for qNMR, the quantitative value and/or absolute purity of the analyte can be determined with metrological traceability to the International System of Units (SI).

Under properly optimized analytical conditions, LC/MS or LC-MS/MS presents the potential for direct quantification of THI in caramel III without the need for sample pretreatment. The absolute purity of a THI analytical standard can be determined using qNMR. In this study, these advanced analytical techniques were combined to accurately determine the content of THI in caramel III commercial products. The objectives of the study were to develop a reliable analytical method for THI in caramel III commercial products, to obtain more reliable SI-traceable analytical data, and to propose an alternative to the JECFA standard method. The THI content in six samples of caramel III commercial products was directly determined using our proposed HILIC-LC/MS method and a THI analytical standard, the absolute purity of which was determined beforehand by qNMR. In addition, we compared the THI content in a sample of caramel III determined using four

different methods: the JECFA standard method, C18-LC/MS, HILIC-LC/MS, and the previously reported revised method.

Ⅱ Materials and Methods

1. Chemicals and Samples

THI was used as an analytical standard in this study and was obtained from the Japan Caramel Industrial Association (JCIA). Six samples of caramel III (ammonia caramel) commercial products (samples 1, 3, and 5 [powder type] and samples 2, 4, and 6 [liquid type; dry solid content of 74.5, 60.4, and 61.9%, respectively]) were obtained from the JCIA and were used as test samples. HPLC-grade acetonitrile and methanol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and 1 mol/L ammonium formate aqueous solution, trifluoroacetic acid (TFA), and 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (DSS-d_6) reference material (certified purity 92.3±0.8% [w/w]) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deuterium oxide (D_2O) was obtained from ISOTEC (Miamisburg, OH, USA). Potassium hydrogen phthalate (PHP) (NMIJ CRM 3001-a, certified purity 100±0.027% [w/w]), which served as a CRM, was obtained from the National Metrology Institute of Japan–National Institute of Advanced Industrial Science and Technology (NMIJ-AIST). Milli-Q water (Millipore, Bedford, MA, USA) was used for analyses.

2. Instruments

qNMR spectra were recorded using a JNM ECA 600 MHz spectrometer (JEOL Ltd., Tokyo, Japan). An ACQUITY UPLC/SQD system (Waters, Milford, MA, USA) was used for LC/MS analyses. An XP2U ultramicrobalance (Mettler Toledo International Inc., Greifensee, Switerland) and an Multipette Xstream electric auto-pipetter (Eppendorf AG, Hamburg, Germany) were used for accurate measurement of weight and volume.

3. Determination of THI absolute purity using qNMR

The qNMR reference solution was prepared by dissolving 5.0 mg of DSS-d_6 in 0.5% TFA D_2O. Calibration of the DSS-d_6 concentration in the qNMR reference solution was carried out as follows. First, precisely 3.0 mg of PHP was dissolved in 1.0 mL of qNMR reference solution, and 0.6 mL of the resulting solution was transferred to an NMR test tube (ϕ 5 mm × 8 in.: Wako Pure Chemical Industries, Ltd.). The concentration of DSS-d_6 in the qNMR reference solution was calculated using the ratio of the signal integral at δ_H 0 ppm (nine protons of DSS-d_6) to that at δ_H 7.69 and 7.82 ppm (four protons of PHP). The DSS-d_6 concentration was calculated as 0.09166 mg/mL according to the following equation (equation 1):

$$C_{DSS}\ (mg/mL) = (M_{DSS} \times I_{DSS} \times H_{PHP} \times W_{PHP} \times P_{PHP}) / (M_{PHP} \times I_{PHP} \times H_{DSS} \times V \times 100) \text{ ------ equation 1.}$$

In equations 1 and 2 (presented below), C represents the concentration, M represents the molecular weight, I represents the signal area, H represents the number of protons associated with the signal, W represents the weight, V represents the volume of the solution, and P represents purity. Subscripts denote the particular substances analyzed.

Next, 1.5 mg of THI was accurately weighed and dissolved in 1.0 mL of qNMR reference solution. The solution was then subjected to qNMR analysis, and the purity of THI was calculated using the ratio of the signal at δ_H 0 ppm (nine protons of DSS-d_6) to that at δ_H 7.65 ppm (one proton of THI). The purity of THI was calculated as 85.1% (w/w) according to the following equation:

$$P_{THI}\ (\%w/w) = (M_{THI} \times I_{THI} \times H_{DSS} \times C_{DSS} \times V \times 100) / (M_{DSS} \times I_{DSS} \times H_{THI} \times W_{THI}) \text{ ------ equation 2.}$$

qNMR analyses were conducted using optimized quantitative acquisition parameters: 5-mm broadband auto-tune probe; probe temperature around 23°C, spectral width of −5 to 15 ppm; auto-filter, 8 times; spectrum data points, 64 K; spectral resolution, 0.25 Hz: number of scans, 16; no spinning; pulse angle, 90°; relaxation delay, 60 s; multi-pulse decoupling with phase and frequency switching (MPF-8) ^{13}C decoupling. The data were processed using Alice 2 for qNMR software (JEOL). The signal integrals were used for quantitation. The chemical shifts were referenced to DSS-d_6 at 0 ppm.

4. Preparation of standard and test solutions

The THI stock solution was prepared by placing 1 mg of THI into a 10-mL measuring flask and adding water to a final concentration of 100 μg/mL. The stock solution was then diluted to 0.02, 0.05, 0.10, 0.20, and 0.40 μg/mL with 0.01 mol/L ammonium formate/acetonitrile (5:95, v/v), and the standard solutions were used to determine the THI content in caramel III commercial products by absolute calibration and standard addition procedures.

The procedure for preparing the test solutions differed slightly for powder-type and liquid-type caramel III products. For powder-type samples, 100 mg of sample was accurately measured and placed into a 25-mL measuring flask and dissolved with methanol/water (90:10, v/v). The solution was then sonicated for 10 min and allowed to stand at room temperature to cool, after which the supernatant was filtered through a 0.45-μm Millex®-LH membrane filter (Merck KGaA, Darmstadt, Germany). The resulting filtrate served as the test solution. For liquid-type samples, 180 mg of sample

was accurately measured and transferred into a beaker. A small amount of methanol/water (90:10, v/v) was added to the flask and the sample was sonicated for about 2 min, after which the suspended solution was poured into a 25-mL measuring flask. This process was carried out three times until the entire sample was transferred to the measuring flask. Subsequent steps were identical to those for the preparation of powder-type products.

For absolute calibration procedure, a portion of each test solution was subjected to LC/MS analysis, and the THI content was determined based on an absolute calibration curve. In the standard addition procedure, 1.0 mL of the standard solution differed from the concentration of THI and the test solution were mixed, filtered through a 0.20-μm Millex®-LG membrane filter (Merck KGaA), and subjected to LC/MS.

5. HILIC-LC/MS analysis of the THI content in caramel III commercial products

Each test solution was subjected to LC/MS analysis using an Atlantis HILIC silica column (2.1 mm i.d × 150 mm; 5-μm particle size; Waters) under the following conditions: LC: column temperature 40°C; mobile phase A = 0.01 mol/L ammonium formate, mobile phase B = acetonitrile; A/B = 5/95 (0-3 min) → 10/90 (15 min); flow rate, 0.2 mL/min; MS: capillary voltage, 3.0 kV; cone voltage, 30 V; source temperature, 110°C; desolvation temperature, 350°C; desolvation gas flow rate, 800 L/h; cone gas flow rate, 30 L/h; detection, ESI, selected ion recording (SIR) mode, THI for *m/z* 231.2 $[M+H]^+$ and *m/z* 229.2 $[M-H]^-$. The content of THI in caramel III commercial products was determined using the absolute calibration and standard addition procedures. The resulting experimentally determined values were finally corrected based on the purity of the THI analytical standard.

Ⅲ Results and Discussion

1. Purity of the THI analytical standard

In order to accurately determine the THI content using the HPLC method, it would be necessary to use a THI CRM, which carries an assurance of absolute purity. However, no THI CRM is currently available on the reagent market. We were, however, able to obtain an analytical standard from a manufacturer of caramel colors. Because the absolute purity of the THI analytical standard provided by the JCIA for this study had not been determined, we determined its absolute purity using qNMR prior to the analysis of the THI content in caramel III commercial products.

We previously reported the development of a qNMR technique designated AQARI (accurate quantitative NMR with internal reference substance), which uses an SI-traceable reference material.[19-21] qNMR is recognized as a promising analytical technique for determining the absolute purity or content of an organic substance because it does not require an identical standard material. The technique is utilized in a variety of fields, such as pharmaceutical sciences, environmental chemistry, and food chemistry.[19, 20, 22, 23] Using a previously reported procedure,[21] we calibrated the concentration of DSS-d_6 in the qNMR reference solution using PHP, which was one of the primary standard materials. The concentration of DSS-d_6, which served as a secondary standard material, was determined as 0.09166 mg/mL in 0.5% TFA D_2O. After a precise amount of THI analytical standard was dissolved in the qNMR reference solution, the absolute purity was calculated from the ratio of the signal area of DSS-d_6 at δ_H 0 ppm to that of the H5 position of THI at δ_H 7.65 ppm. The proton signal of the H5 position on the imidazole ring of THI was suitable for THI quantification by qNMR, as it provided a singlet signal with no impurity signals in the integrated area. The absolute purity of THI was thus determined as 85.1% (w/w) (average of duplicate measurements, SD 0.05% [w/w]) and was indirectly SI-traceable through the PHP primary reference material.

No significant chromatographic peaks derived from impurities were observed in HPLC/UV analysis of the THI analytical standard, suggesting that the THI analytical standard was in the salt or hydrate form. This result showed that the absolute purity of the THI analytical standard used for accurate quantification by chromatography should be determined and that the bias reflected in the quantitative values should be corrected for based on the purity.

2. Direct analysis of THI content in caramel III using HILIC-LC/MS

We evaluated both HPLC/UV and LC/MS in development of a method for direct determination of the THI content in caramel III commercial products. In evaluations of several HPLC column types with respect to retention and separation of THI, poor results were obtained with C18 reverse-phase columns. In contrast, HILIC columns provided appropriate separation for the analysis of THI in caramel III products. Among several HILIC column tested, the Atlantis HILIC silica column provided the best separation and peak shape.

We further optimized the LC/MS conditions, such as the ratio of mobile phase solvents for peak separation and mass detector parameters for THI ionization. Under the optimized HILIC-LC/MS conditions, THI produced a protonated molecule ($[M+H]^+$ *m/z* 231.2) and a deporotonated molecule ($[M-H]^-$ *m/z* 229.2). A typical SIR chromatogram of a caramel III sample is shown in Fig. 2. The THI peak shows a retention time of 6.8 min. Negative-mode SIR detection was selected for quantification of THI because the baseline was flatter in

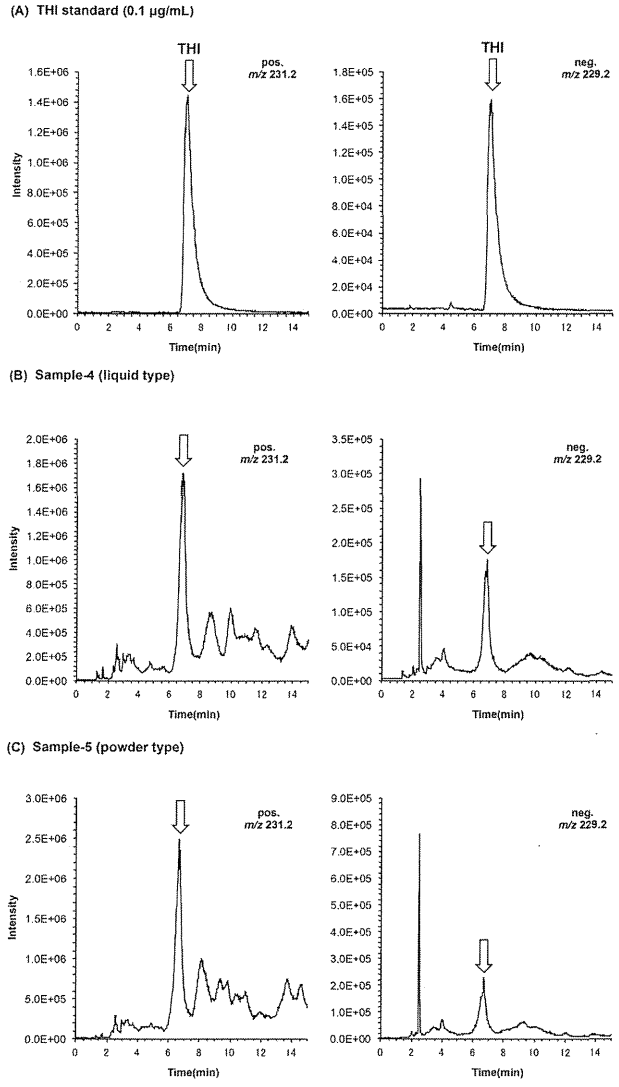

Fig. 2. HILIC-LC/MS chromatograms of the THI standard and test solutions
(A) 0.1 μg/mL THI; (B) sample 4: liquid-type product; (C) sample 5: powder-type product

negative mode than in positive mode.

To validate the optimized HILIC-LC/MS method, we evaluated various experimental parameters, such as linearity, limit of detection (LOD), and limit of quantification (LOQ). The calibration curves were linear over the concentration range 0 to 0.4 μg/mL for the THI standard solutions, and the correlation coefficient (r^2) was >0.99 for the absolute calibration procedure and >0.97 for the standard addition procedure. The LOD was determined based on the lowest concentration with a signal to noise (S/N) ratio of >10, and the LOQ was defined as 2 times the LOD. The LOD and LOQ values were 0.29 μg/g and 0.57 μg/g for powder-type samples and 0.16 μg/g and 0.32 μg/g for liquid-type samples, respectively.

The THI content in six samples of caramel III commercial products was determined using the absolute calibration and standard addition procedures with the HILIC-LC/MS method and THI analytical standard, the absolute purity of which had been previously determined by qNMR. Test solutions were diluted and filtered without tedious pretreatment (e.g., SPE and DNPH derivatization). The THI content in each of the powder- and liquid-type samples is shown in Table 1. The THI content in the powder-type samples ranged from 20.2 to 29.4 μg/g as determined using the standard addition procedure and from 18.4 to 27.4 μg/g as determined using the absolute calibration procedure. The THI content of the liquid-type samples ranged from 6.3 to 21.6 μg/g as determined using the standard addition procedure and 3.6 to 21.0 μg/g as determined using the absolute calibration procedure. Analyses carried out using the absolute calibration procedure indicated a slightly lower THI content than those carried out using the standard addition procedure, suggesting that a matrix effect influences analyses using the absolute calibration procedure. All quantitation values were lower than the JECFA and Japanese specified limit for THI in caramel III, 40 μg/g.

Table 1. Contents of THI in caramel III commercial products determined by HILIC-LC/MS

Sample No.	Type	Content of THI (μg/g)[a]	
		Negative mode $[M-H]^-$	
		Absolute calibration	Standard addition
Sample 1	Powder	21.1 (0.9)	24.9 (1.4)
Sample 2	Liquid	3.6 (0.1)	6.3 (1.0)
Sample 3	Powder	18.4 (1.8)	20.2 (3.0)
Sample 4	Liquid	14.4 (0.3)	17.6 (1.6)
Sample 5	Powder	27.4 (2.2)	29.4 (3.0)
Sample 6	Liquid	21.0 (0.7)	21.6 (1.1)

a) The contents are shown as the average with SD in parentheses (n = 3).

3. Comparison of THI content as determined by different methods

We previously reported a revised HPLC/UV method employing DNPH derivatization for the quantification of THI in caramel III commercial products and demonstrated the superiority of the revised method to the JECFA standard method. To evaluate the reliability of the method proposed in the present report, the THI content in sample 5 was quantified using the JECFA standard method, the previously reported revised method, the C18-LC/MS method, and the current HILIC-LC/MS method, and the results were compared. The experimental conditions are shown in Table 2.

As shown in Table 2, the JECFA standard method recommends the use of a classical 10-μm particle size C8 column (LiChrosorb RP-8, 4.6×250 mm, 10 μm) for analysis of the THI-DNPH derivative. The peak separation provided by the revised method using a modern 5-μm particle size C8 column was improved relative to that obtained using the

Table 2. Comparison of THI contents in sample-5 quantified by official, revised, C18-LC/MS and HILIC-LC/MS methods

Method	Measurement target	Detection	Column	Quantitation procedure	THI content	
					AV μg/g	S.D.
Official DNPH derivative C8-HPLC/UV[a) b)]	DNPH-THI[e)]	UV	LiChrosorb RP-8, 4.6×250 mm, 10 μm	absolute calibration	24.9	(1.6)[f)]
Revised DNPH derivative C8-HPLC/UV[a) c)]	DNPH-THI[e)]	UV	TSKgel Octyl-80Ts, 4.6×250 mm, 5 μm	absolute calibration	30.0	(1.7)[f)]
	DNPH-THI[e)]	UV	Wakosil-II 5C8 HG, 4.6×250 mm, 5 μm	absolute calibration	27.5	(0.7)[f)]
C18-LC/MS[a) d)]	DNPH-THI[e)]	MS	L-column2 ODS, 2.1×150 mm, 3 μm	absolute calibration	30.2	(1.6)[f)]
	DNPH-THI[e)]	MS	Inertsil ODS-4, 2.1×150 mm, 3 μm	absolute calibration	31.3	(1.6)[f)]
	DNPH-THI[e)]	MS	TSKgel ODS-100V, 2.0×150 mm, 3 μm	absolute calibration	31.9	(1.5)[f)]
HILIC-LC/MS	THI	MS	Atlantis HILIC Silica, 2.1×150 mm, 5 μm	absolute calibration	27.4	(2.2)
	THI	MS	Atlantis HILIC Silica, 2.1×150 mm, 5 μm	standard addition	29.4	(3.0)

a) The results were in our previous report.[9)] b) JECFA standard method. HPLC/UV conditions: flow rate, 0.7 mL/min; mobile phase, 0.1 M H_3PO_4/MeOH (50:50, v/v); column temp., 30°C; detect., UV 385 nm. c) Our revised method. HPLC/UV conditions: flow rate, 0.8 mL/min; mobile phase, 0.1 M H_3PO_4/MeOH (70:30, v/v); column temp., 30°C; detect., UV 385 nm. d) LC/MS conditions: flow rate, 0.2 mL/min; mobile phase, 0.1% HCOOH/MeOH (70:30, v/v); column temp., 30°C; detect., ESI pos. SIR mode, *m/z* 411. e) DNPH derivatization and pretreatment were according to JECFA standard method. f) The value was not corrected by the absolute purity of THI analytical standard.

JECFA standard method, resulting in higher quantitation with the revised method. The THI values determined using the revised method were similar to those obtained using the C18-LC/MS method (Table 2). These results were similar to those for direct analysis of THI in caramel III commercial products obtained using the HILIC-LC/MS method, indicating a THI content of around 30 μg/g, higher than the 24.9 μg/g value obtained using the JECFA standard method. The HILIC-LC/MS method allows for direct determination of THI content without the need for SPE or derivatization pretreatment. In addition, the absolute purity of the THI analytical standard was indirectly determined by qNMR with SI traceability through the CRM PHP. Therefore, we conclude that THI content data obtained using the HILIC-LC/MS method are more reliable than data obtained using the other methods examined in this study. Our data suggest that the previously reported revised method and the HILIC-LC/MS method described here provide more accurate data regarding the THI content of caramel III commercial products. Our findings also suggest that the JECA standard method for analysis of THI in caramel III should be improved.

Ⅳ Conclusion

The JECFA standard method for determination of the THI content in caramel III commercial products does not require the use of expensive equipment, such as LC/MS instruments. However, the JECFA standard appears to provide less credible data, because the HPLC column recommended does not provide adequate separation of the THI peak from those of other constituents in caramel III. We previously reported a revised method using a modern C8 column that provides better separation for the determination of THI-DNPH. The improved peak separation afforded by the revised method yielded higher THI content values than the JECFA standard method. However, the complex pretreatment and derivatization steps required in the revised method raised concerns regarding the accuracy of results. In the present study, we therefore developed a simple analytical method using HILIC-LC/MS for the direct determination of THI. The proposed HILIC-LC/MS method can be used to directly determine the THI content in caramel III products through the use of a THI analytical standard, the absolute purity of which is determined beforehand with SI traceability by qNMR. Six samples of caramel III commercial products in Japan were analyzed in this study using the proposed HILIC-LC/MS method. The THI content of each of the samples was lower than the officially stipulated limit. Similar values for THI content (around 30 μg/g) were observed when comparing samples analyzed using the HILIC-LC/MS method, the previously reported revised method, and the C18-LC/MS method. This value was higher than the 24.9 μg/g determined using the JECFA method. We therefore conclude that the JECA standard method should be improved in terms of the recommended analytical conditions. The proposed HILIC-LC/MS method and the previous revised method represent alternatives to the JECFA standard method for determining THI content.

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論　　文

HILIC-LC/MS を用いたカラメル III 中の 2- アセチル -4- テトラヒドロキシブチルイミダゾールの直接定量分析法の開発

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キーワード：HILIC、LC/MS、qNMR、カラメル、2- アセチル -4- テトラヒドロキシブチルイミダゾール

概　要

親水性相互作用クロマトグラフィー（HILIC）カラムを用いた LC/MS（HILIC-LC/MS）によるカラメル III 中の不純物 2- アセチル -4- テトラヒドロキシブチルイミダゾール（THI）の直接定量分析法を開発した。本研究では、予め定量 NMR により絶対純度を求めた 2- アセチル -4- テトラヒドロキシブチルイミダゾールを定量用標準品とし、絶対検量線法及び標準添加法によりカラメル III 中のより信頼性の高い正確な THI 含量を求めた。現在、JECFA では、カラメル III 中の THI の定量分析法として、2,4- ジニトロフェニルヒドラジンにより誘導体化した THI を粒子径 10 μm の C8 カラムを用いて HPLC/UV で分析する方法が設定されている。JECFA 及び我々が開発した HILIC-LC/MS により、同試料について求めた定量値を比較した結果、JECFA による定量値は夾雑物との分離が不十分なため、HILIC-LC/MS による定量値より低い値を示した。この結果は、JECFA の規定する分析条件には正確な定量値を得るための改良の余地があることを支持するものである。

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Confirmation of the configuration of two glucuronic acid units in glycyrrhizic acid

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Abstract

Glycyrrhizic acid (GA), a triterpenoid saponin containing two glucuronic acid (GlcA1 and GlcA2) units, is found in the roots of *Glycyrrhiza* plants, and has been widely used as a natural sweetener for foods as well as a natural medicine. Purified GA is commercially available from various manufacturers as an analytical standard or a biochemical reagent. While producers describe the configurations of GlcA1 and GlcA2 as α and β-forms, respectively, reports of the structural elucidation of GA have proposed that both GlcA units are β-form. To clarify this point, commercial GA from various sources was analyzed by 1D and 2D NMR studies. Results confirmed that the actual configuration of both GlcA units in GA is β-form.

Keywords : glycyrrhizic acid, glucuronic acid, natural sweetener

I Introduction

Glycyrrhizic acid (GA) is a triterpenoid saponin found in *Glycyrrhiza* plants such as *Glycyrrhiza glabra* (licorice), *G. inflata*, and *G. uralensis*. Since GA is 30–50 times sweeter than sucrose, root extracts of *Glycyrrhiza* (known as licorice root extract) have been used as a natural sweetener for foods[1]. In addition, it has been extensively reported that GA has several pharmacological activities, including anti-inflammatory[2], immunomodulatory[3], anti-ulcer[4] and anti-tumorigenic effects[5, 6]. Moreover, licorice root is a well-known oriental and occidental herbal medicine that is frequently prescribed for the treatment of various diseases. Purified GA is commercially available, and is utilized as an authentic standard in natural medicines and as a research agent for the investigation of biochemical and pharmaceutical activities.

GA is composed of a triterpenoid aglycone, glycyrrhezinic acid (GLA), and two glucuronic acid units (GlcA1 and GlcA2). The two GlcA units are connected *via* a 1 → 2 glycoside linkage (Fig. 1) and the GlcA1 connects to position 3 of the aglycone GLA *via* a glycoside linkage. With respect to the configuration of the two GlcA units, recent papers dealing with GA prepared from *Glycyrrhiza* plants proposed that both were β-form[7, 8]. However, we noted that the labels of commercial GA and GA salts indicate the configurations of GlcA1 and GlcA2 as α-form and β-form, respectively. In our investigation of commercial reagent labeling, all commercial GA and GA salts were labeled as containing the α-form configuration of GlcA1 in their product catalogues. The origin of the labeling might be an authentic database such as the

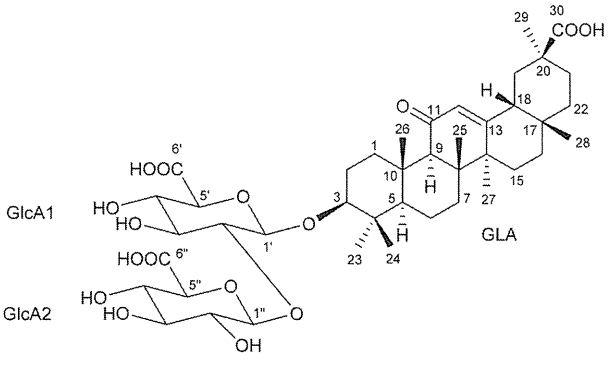

Fig. 1. Structure of glycyrrhizic acid (GA)

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Chemical Abstract Service (CAS) that states that the structure of GlcA1 is α-form, e.g., GA (CAS Registry Number: 1405-86-3), GA ammonium salt (CAS Registry Number: 53956-04-0), and GA dipotassium salt hydrate (CAS Registry Number: 68797-35-3). These inconsistencies create confusion in analytical and biochemical investigations using commercial GA reagents as authentic standards.

In order to clarify the situation, we present the correct structural determination of commercial GA reagents purchased from various distributors using high-resolution NMR analysis.

Ⅱ Material and Methods

Reagents: Two GA reagents and five GA ammonium salt reagents were purchased from seven sources as follows: glycyrrhizic acid standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan), glycyrrhizic acid (Tokyo Chemical Industry co., Ltd., Tokyo Japan), glycyrrhizic acid monoammonium salt (Sigma-Aldrich, MO, USA), glycyrrhizic acid monoammonium salt standard (Kanto Chemical Co., Inc., Tokyo, Japan), glycyrrhizic acid ammonium salt, trihydrate (LKT Labs, Inc., MN, USA), glycyrrhizic acid ammonium salt (ChromaDex, Inc., CA, USA), and glycyrrhizic acid monoammonium salt (Acros Organics, Geel, Belgium). Commercial catalogues of these sources noted the configurations of GlcA1 and GlcA2 in all reagents as α-form and β-form, respectively.

NMR study: 1D (^{1}H and ^{13}C) and 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC, and NOESY experiments) spectra of GA were recorded on JEOL ECA instruments (600 MHz) in CD_3OD as the solvent at 25°C. The methyl proton signal at 3.30 ppm in CD_3OD was referenced on the NMR measurement.

Ⅲ Results

Seven commercial GA or GA ammonium salts were analyzed using NMR in this study. The commercial catalogues supplied by seven sources indicated the configuration of GlcA1 in GA as α-form, and that of GlcA2 as β-form. To confirm the configuration of GlcA units in GA, the standard GA reagent purchased from Wako Pure Chemical Industries was first analyzed using NMR. The chemical shifts (δ_H and δ_C) of GA were recorded in CD_3OD at 25°C and all signals were assigned by the analysis of 1D (^{1}H and ^{13}C) and 2D NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC, and NOESY experiments). The ^{13}C NMR spectrum of GA showed 42 signals, including a typical ketone signal (δ_C 202.7) corresponding to C11 on GLA and three carboxyl signals (δ_C 180.4, 172.6 and 172.1) corresponding to C30, C6′ and C6″, respectively (Table 1). The aglycone GLA is known to be an 18 β-H-oleanane-type compound (18β-GLA)[9]. The 18α-epimer

Table 1. Chemical shifts of glycyrrhizic acid (GA) in CD_3OD

position	δ^1H (multiplet, J Hz)	δ^{13}C
GLA		
1	0.99 (td, 13.0, 3.5) 2.66 (dt, 10.3, 3.5)	40.2
2	1.74 (m) 1.81 (m)	27.0
3	3.16 (dd, 11.7, 4.4)	90.8
4		40.6
5	0.76 (brd, 12.0)	56.5
6	1.43 (m) 1.60 (br, 12.0)	18.4
7	1.43 (m) 1.72 (m)	33.8
8		46.7
9	2.43 (s)	63.1
10		38.0
11		202.7
12	5.56 (s)	128.9
13		172.4
14		44.6
15	1.04 (m) 1.24 (brd, 14.0)	27.6
16	1.87 (m) 2.14 (td, 13.8, 4.5)	27.4
17		33.0
18	2.18 (dd, 13.5, 3.5)	50.0
19	1.70 (m) 1.83 (m)	42.4
20		44.9
21	1.39 (m) 1.94 (m)	32.0
22	1.39 (m) 2H	39.0
23	0.81 (s) 3H	16.8
24	1.04 (s) 3H	28.2
25	1.12 (s) 3H	17.0
26	1.12 (s) 3H	19.3
27	1.42 (s) 3H	23.8
28	0.82 (s) 3H	29.2
29	1.16 (s) 3H	28.7
30		180.4
GlcA1		
1′	4.51 (d, 7.5)	105.3
2′	3.50 (t, 9.5)	84.0
3′	3.58 (t, 9.8)	77.4
4′	3.54 (t, 9.8)	72.9
5′	3.76 (d, 9.0)	76.3
6′		172.6
GlcA2		
1″	4.62 (d, 7.7)	106.3
2″	3.28 (m)	76.3
3″	3.38 (t, 9.8)	77.2
4″	3.52 (t, 9.5)	73.1
5″	3.74 (d, 9.7)	77.6
6″		172.1

of GLA was previously prepared from 18β-GLA and its derivatives were intensively synthesized and investigated for various pharmaceutical activities, including anticancer activity[9]. The isomerization from 18β-epimer to 18α-epimer proceeded under an alkaline condition, which implies a possibility of contamination of 18α-isomer in GA reagents. Therefore, this study attempted to confirm the entire structure of GA, including GLA, using NMR.

To confirm the aglycone GLA structure, the detailed structure of GLA was analyzed by 2D NMR experiments. The ^{1}H-^{13}C HMQC experiment correlated all proton signals with the corresponding 21 carbon atoms. As shown in Fig. 2, the cross peaks observed in the COSY experiment gave five spin-spin systems (H1-H3, H5-H7, H15-H16, H18-H19, and H21-H22). The ^{1}H-^{13}C HMBC correlations of seven singlet methyl signals (H23, H24, H25, H26, H27, H28, and H29) allowed the connection of these methyl groups to adjacent quaternary carbons (C4, C8, C10, C14, C17, and C20). Subsequently, the quaternary carbons were connected to the spin-spin systems by the HMBC correlations to yield two six-membered rings, namely the A and E rings (Fig. 2). The HMBC correlations from a singlet methine signal (H9) to C8, C10, and C11 revealed a bridge connection of two quaternary carbons of C8 and C10 *via* C9 to yield B rings, and further indicated the ketone (C11) to be located adjacent to C9 (Fig. 2). The HMBC correlations from a singlet olefin signal (H12) to the ketone C11 and to a quaternary olefinic carbon (C13) constructed the connection from C11 to C13. This connection was further extended to the quaternary carbon (C14) by the HMBC correlation of the methyl signal (C27) to C13 to yield a 6/6/6 ring system, namely the A, B, and C rings. The HMBC correlation from H12 to C18 showed a connection between C13 and C18 to make ring D. Finally, a carboxylic acid (C30) was connected to the quaternary carbon (C20) on the E ring by the HMBC correlations from the adjacent methyl signal (H29) to C30. Thus, the planar structure of GLA was confirmed to be a triterpene structure containing five rings (Fig. 2). Subsequently, the stereochemistry of GLA was elucidated from the NOESY correlations and coupling constants (Fig. 3). The NOESY correlations around the A and B rings (H1a/H3, H3/H5, H1a/H5, H1a/H9, and H5/H9) indicated axial orientations of all these protons, which means that the hydroxyl group at C3 has a β-orientation. The equatorial position of the C23 methyl group was confirmed by a NOESY correlation observed between H3 and H23. The large coupling constant ($J_{H18,H19\alpha}$ = 13.5 Hz) indicated an axial orientation at C18 on the E ring. The NOESY correlations between H18 and H28 showed the *cis*-form of H18 and the methyl group (C28). In addition to these data, the NOESY correlations between H12 and H18 indicated the β-orientation of H18. The NOESY correlation between H29 and H16 showed that the carboxylic acid (C30) had an axial orientation, as with H18. These results confirmed that the aglycone of GA was 18β-glycyrrhezinic acid (18β-GLA).

To confirm the structure of the glucuronic acids (GlcA1 and GlcA2), the structure of GlcA1 and GlcA2 were analyzed by 2D NMR experiments and from the J values from their ^{1}H NMR spectra. The spin-spin systems starting from the anomeric proton (H1′) at δ_H 4.51 to H5′ at δ_H 3.76 in the GlcA1 unit was assigned by the COSY experiment (Fig. 2). The presence of a carboxylic acid of GlcA1 was confirmed by the HMBC correlation from H-5′ to a carboxyl carbon (C6′) at δ_C 172.6 (Table 1). The signal of H1′ was split into a doublet and showed a coupling constant ($J_{H1',H2'}$) of 7.5 Hz (Table 1). Generally, anomeric configurations are assigned from the magnitude of $J_{1,2}$ with values of 7-9 Hz for the diaxial coupling associated with a β-anomers, while 2-4 Hz is indicative of the equatorial-axial coupling of α-anomers[10]. Furthermore, the coupling constants of other oxymethin protons, such as $J_{H2',H3'}$, $J_{H3',H4'}$, and $J_{H4',H5'}$, were observed to be around 9.5 Hz (Table 1). These values were sufficiently large to assure axial orientations for the five protons (H1′ ~ H5′), allowing a chair form of a pyranosyl ring of GlcA1 (Fig. 3). This interpretation was also supported by the NOESY

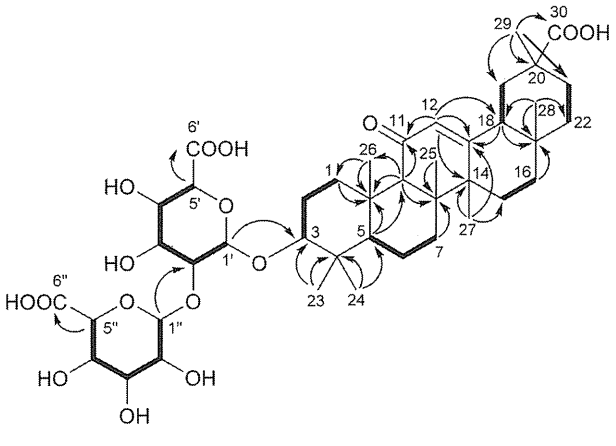

Fig. 2. Key correlations of ^{1}H-^{1}H COSY (bold lines) and ^{1}H-^{13}C HMBC (arrows) of GA

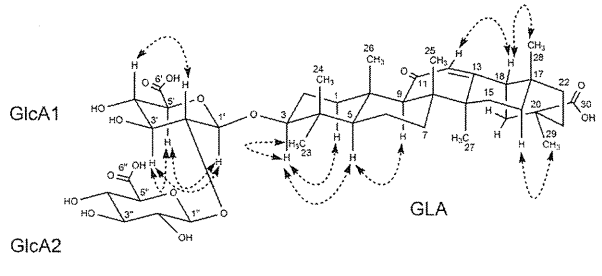

Fig. 3. Structure of GA and NOESY correlations

correlations for H1′/H3′, H1′/H5′, H3′/H5′, and H2′/H4′ (Fig. 3). Thus, the glycosidic bond at C1′ in the glucuronopyranosyl structure of GlcA1 is oriented equatorially and this result definitely indicated that GlcA1 is β-form, not α-form. The glycosidic linkage from the anomeric proton to the aglycone GLA was confirmed by the HMBC correlations from H1′ to C3 (Fig. 2). The structure of glucuronic acid, GlcA2, was also confirmed in a similar manner to GlcA1. An anomeric proton (H1″) observed at δ_H 4.62 also had a large coupling constant (7.7 Hz), confirming the β-form of GlcA2, as with GlcA1 (Fig. 4). The inter-glycosidic linkage was confirmed to be a β1 → 2 link by the HMBC correlation from H1″ to C2′ at δ_C 84.0 of GlcA1 (Fig. 2). The chirality of GlcA units were determined to be both D-form, because previous studies indicated that both units were D-form and furthermore the presence of L-GlcA has not been reported from any natural sources. Based on the foregoing evidence, we concluded that the structure of GA is 3β-hydroxy-11-oxo-18βH-olean-12-en-30-oic acid 3-*O*-[β-D-glucuronopyranosyl-(1 → 2)-β-D-glucuronopyranoside].

In six other commercial GA or GA ammonium salt reagents, the configurations of the two GlcA units were both β-form, as observed with the GA from Wako (Fig. 4). Two anomeric protons of GlcA1 and GlcA2 showed identical coupling constants in each GA or GA ammonium salt, although there were slight differences in chemical shifts between GA and GA ammonium salts (Fig. 4).

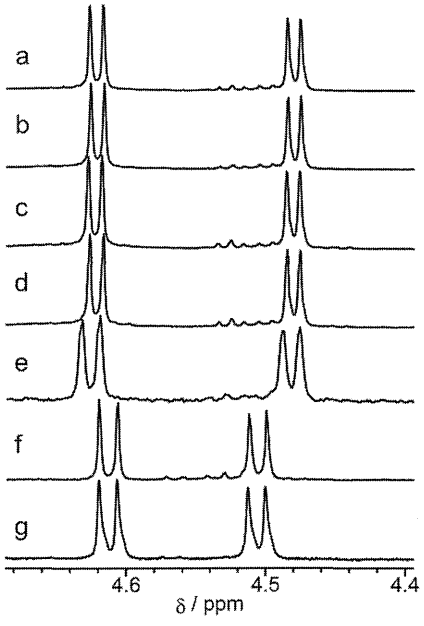

Fig. 4. ^{1}H NMR spectra (600 MHz) around anomeric proton signals of GlcA1 and GlcA2 of seven GA reagents in CD_3OD

Five spectra (a ~ e) are GA ammonium salts, while the remaining two spectra (f and g) are GA.

Ⅳ Discussion

The present NMR studies demonstrated that GlcA1 and GlcA2 were both β-form in the seven GA and GA ammonium salt reagents purchased from seven sources. These results clearly indicated that the supplier information on GA structure is incorrect. We have little information as to why this misinterpretation of the GA structure has occurred. In 1950, Lythgoe and Trippett first identified two hexuronic acids in GA as GlcA units[11]. Furthermore, they proposed that the stereochemistry of GlcA units was one β-link as the internal glycosidic bond and the other as an α-link; this was determined by comparison of $[\alpha]_D$ values between a permethylate derivative of GlcA units prepared from GA and authentic glycosidic compounds. This interpretation was generally accepted, and the α-configuration of GlcA1 in GA was taken as correct. After approximately 40 years, however, Khalilov *et al.* revealed the configuration of GlcA1 to be β-form based on an ^{13}C-NMR study of GA purified from natural sources[12]. Report of the revised structure was subsequently followed by an advanced NMR study on GA[13]. During research on natural products in *Glycyrrhiza* plants, GA was often isolated as a by-product and its structure was elucidated[7, 8]. These reports also showed that the GlcA1 configuration of the isolated GA is β-form. Furthermore, the X-ray crystal structure of GA dipotassium salt was recently analyzed to evaluate the coordination system of potassium ions to GA, which subsequently indicated GlcA1 to be β-form[14, 15]. Accordingly, recent research has determined the GlcA1 configuration in GA to be β-form. Nevertheless, commercial reagent catalogues and chemical databases, including package inserts for drugs, designated GlcA1 in GA as α-configuration, which has led to confusion in the research areas of analytical chemistry and biochemistry.

GA is one of the most well known and successful natural sweeteners, and is also used as a phytomedicine. Moreover, numerous biochemical and chemical studies dealing with GA have been reported. Yet, surprisingly, its incorrect structure continues to be used in commercial catalogue product information. Furthermore, since the incorrect catalogue information is likely to be recognized as the standard structure of the compound, this might lead to misinterpretation of research results. We believe that this study definitively clarifies this misinformation, and we urge the rapid revision of the incorrect structure of GA in commercial catalogues and other literature.

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論　　文

グリチルリチン酸に含まれるグルクロン酸の立体化学の確認

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キーワード：グリチルリチン酸、グルクロン酸、天然甘味料

概　要

グリチルリチン酸（GA）は 2 つのグルクロン酸（GlcA1 と GlcA2）を含むトリテルペン型サポニンである。GA はカンゾウ属（*Glycyrrhiza*）の植物の根に含まれている。生薬として、また食品に添加する天然甘味料として長く用いられてきた。精製された GA は分析用の標準品または生化学試薬として複数の試薬会社から入手が可能である。試薬会社のカタログには、GlcA1 の立体化学はα型で、一方の GlcA2 はβ型と記されている。Chemical Abstract においても、GlcA1 はα型で GlcA2 はβ型とされている。しかしながら近年の研究では、2 つの GlcA はともにβ型との報告が続いている。この混乱を解決すべく、複数の試薬会社から高純度に精製された GA または GA 塩の試薬を入手し、1 次元および 2 次元 NMR によって構造を詳細に解析した。その結果、2 つの GlcA はともにβ型であることが確認された。

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Analytical Methods

Metabolomics approach of infant formula for the evaluation of contamination and degradation using hydrophilic interaction liquid chromatography coupled with mass spectrometry

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ABSTRACT

In this study including the field of metabolomics approach for food, the evaluation of untargeted compounds using HILIC–ESI/TOF/MS and multivariate statistical analysis method is proposed for the assessment of classification, contamination and degradation of infant formula. HILIC mode is used to monitor more detected numbers in infant formulas in the ESI-positive scan mode than the reversed phase. The repeatability of the non-targeted contents from 4 kinds of infant formulas based on PCA was less than the relative standard deviation of 15% in all groups. The PCA pattern showed that significant differences in the classification of types and origins, the contamination of melamine and the degradations for one week were evaluated using HILIC–ESI/TOF/MS. In the S-plot from the degradation test, we could identify two markers by comparison to standards as nicotinic acid and nicotinamide. With this strategy, the differences from the untargeted compounds could be utilized for quality and safety assessment of infant formula.

1. Introduction

Infant formula has been recognized as a very important commodity regarding food quality and safety. Additives, nutrients and natural contents are included in infant formulas depending on the various kinds of earmark products worldwide. On a global mass scale, infant formula would be standardized regarding its definitions and analytical methods of existing additives, nutrients and natural contents for food quality and safety. However, in 2008, the highest contamination of melamine was detected in infant and/or follow-up formula foods for children (Tyan, Yang, Jong, Wang, & Shiea, 2009; Xin & Stone, 2008). At that time, unexpected melamine that contains a high percentage of nitrogen to make the protein content of food appear higher than the actual value could not be determined before limiting the extent of damage. It was impossible to predict the artificially enhance protein concentrations using unknown nitrogen-rich compounds in infant formula products (Abernethy & Higgs, 2013; MacMahon, Begley, Diachenko, & Stromgren, 2012). Later on, the nitrogen-rich dicyandiamide was also detected in infant formula (Inoue, Sakamoto, Min, Todoroki, & Toyo'oka, 2014). Thus, we prevent a future recurrence of unexpected accidents of infant formula using untargeted metabolomics strategy.

Based on the evaluation of the unexpected contents for food quality and safety, various ideas have been to use protein oxidation, antioxidant, nutrition and environments (Friel et al., 2013; Heller, Keoleian, & Willett, 2013; Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008; Zhang, Xiao, & Ahn, 2013). Recently, the analytical strategy for non-targeted food ingredients is a major topic in modern Food Analytical Science, as demonstrated by the growing activity in the very new field of *Foodomics* included food metabolomics that is defined as a discipline that studies the food and nutrition domains through the application of *Omics* technologies (Cifuentes, 2009; García-Cañas, Simó, Herrero, Ibáñez, & Cifuentes, 2012; Herrero, Simó, García-Cañas, Ibáñez, & Cifuentes, 2012). A broad vision means not only a broad expertise acquisition, but also the ability and possibility of resolving any unexpected food accidents using metabolomics approach (Cevallos-Cevallos & Reyes-De-Corcuera, 2012). However, due to the unexpected accidents of infant formula, we are still far from an approach that integrates the untargeted metabolomics approach. A chemical

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fingerprint tends to be focused on the application of an interesting method based on the metabolomics approach in human milk (Marincola et al., 2012). The untargeted metabolomics approach to simultaneously measure dynamic changes of many compounds in various food samples has been utilized as high resolution nuclear magnetic resonance (NMR) and mass spectroscopy (MS) coupled with either high or ultrahigh resolution liquid (LC) or gas (GC) chromatographic technique. Compared to many techniques, LC/MS is more sensitive and allows for the measure of a broader array of metabolites in food and/or biological samples (Roux, Lison, Junot, & Heilier, 2011; Toyo'oka, 2008; Rogachev & Aharoni, 2012). LC/MS applications in the metabolomics field have been based on reversed-phase (RP) chromatographic methods, in which only non-polar and medium polarity analytes can be separated and retained on RP columns. On the other hand, hydrophilic interaction chromatography (HILIC) offers a different selectivity, with better retention of polar analytes not easily retained or indeed not retained at all using RP columns (Kawachi et al., 2011). Since infant formula contains about high-percentage constituent of water, many contents are expected to be highly polar and to be better separation of chemical status using HILIC than RP modes. However, there are only a very limited number of studies that have comprehensively performed the coverage and endurance of HILIC method for untargeted, complicated and exhaustive analytes of infant formula. Thus, our LC/MS assay based on HILIC mode can obtain the large number of chromatogram data (*m/z*, retention time, peak response and detected numbers) from infant formulas for multivariate statistical analysis of untargeted chemicals. Also, due to our approach, this assay are established based on different patterns that showed the visualized integration of data and chemicals that are statistically significant such as kinds, origin, contamination and degradation in infant formula with steady-state homeostasis. Therefore, the chemical profiling and/or fingerprinting of different patterns under steady-state conditions has been demonstrated, and is opening new possibilities for the assessment of infant formulas based on a non-targeted metabolomics approach. In this study, we propose the unique approach of *Foodomics* included metabolomics that the assay of non-targeted low-molecular-weight compounds in infant formulas by HILIC coupled with electrospray time-of-flight MS (HILIC–ESI/TOF/MS) and a multivariate statistical analysis that was applied to the assessment of the unexpected contaminations and degradations.

2. Material and methods

2.1. Reagents and solutions

Melamine was purchased from the Kanto Chemical Co. (Tokyo, Japan). Methanol, acetonitrile, formic acid, ammonium acetate, nicotinic acid and nicotinamide were obtained from the Wako Chemical Co. (Osaka, Japan). For the mobile phase, pure water, acetonitrile and methanol were used from Merk KGaA (Billeria, MA, USA). All other chemicals were of analytical grade. Deionized and distilled water was used throughout the study (Aquarius PWU200 automatic water distillation apparatus, Advantec, Tokyo, Japan). The infant formula samples were obtained from local stores in Japan, China and other countries.

2.2. LC instrument and conditions

The LC system was a Waters Acquity H Class from Waters Co. (Milford, MA, USA). The RP and HILIC separations were performed using an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm), TSKgel NH_2 column (3.0 μm, 2.1 × 150 mm) and TSKgel Amide-80 column (2.0 μm, 2.0 × 150 mm) at 40 °C. The injection volume was 5 μL. The mobile phase consisting of solvent A; 10 mM ammonium acetate in water, and solvent B; acetonitrile, was delivered at the flow rate of 0.2 or 0.4 mL/min. Three gradient modes of this mobile phase were used for the simple separation of the reversed phase (Type A), HILIC for NH_2 mode (Type B) and HILIC for Amide-80 mode (Type C) of the non-targeted compounds in the infant formulas. The gradient elution of Type A was as follows: 0.0 min [A/B: 98/2], 3.0 min [A/B: 98/2], 30 min [A/B: 2/98], 35.0 min [A/B: 2/98], 35.5 min [A/B: 98/2] and 50.0 min [A/B: 98/2] delivered at the flow rate of 0.2 mL/min. The gradient elution of Type B was as follows: 0.0 min [A/B: 2/98], 3.0 min [A/B: 2/98], 30 min [A/B: 98/2], 35.0 min [A/B: 98/2], 35.1 min [A/B: 2/98] and 50.0 min [A/B: 2/98] delivered at the flow rate of 0.2 mL/min. The gradient elution of Type C was as follows: 0.0 min [A/B: 2/98], 1.0 min [A/B: 2/98], 11 min [A/B: 90/10], 12.0 min [A/B: 90/10], 12.1 min [A/B: 2/98] and 18.0 min [A/B: 2/98] delivered at the flow rate of 0.4 mL/min.

2.3. MS instrument and conditions

The separated compounds were detected by a Waters LCT Premier XE time-of-flight mass spectrometer (TOF/MS) from Waters Co. (Milford, MA, USA). The electrospray (ESI) (positive ionization mode) conditions were as follows: capillary voltage was 3.0 kV, sample cone was 15 V, source temperature of 120 °C and desolvation temperature of 350 °C. The cone and desolvation gas flows were 50 and 650 L/h, respectively, and were obtained flowing nitrogen. The analytical mode and dynamic range were the V mode and normal, respectively. The aperture 1 voltage was 15 V. For calibration, the reference solution used 4 μg/mL leucine enkephalin (*m/z* 556.28, 2 ppm) in 0.1% formic acid in water/acetonitrile (5/5, v/v). The scan mode was used from *m/z* 100 to 1000.

2.4. Sample preparation

The 0.5 g samples were weighed in plastic tubes. Five-mL of water was then added and mixed. The infant formula samples were pretreated using Amicon Ultra-4 (Ultracel-3K, regenerated cellulose 3000 M.W. for volumes <4 mL, Milliore Co., Ltd., Billerica, MA, USA). The 0.5 mL sample solution was eluted through this cartridge by centrifuging at 14,000*g* for 10 min, added of 0.5 mL acetonitrile, filtered through a 0.2 μm filter for LC and measured by LC/MS assay.

2.5. Multivariate statistical analysis

The LC/MS data were analyzed for peak detection and alignment from *m/z* 100 to 1000, and exported for the principal components analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) by a MarkerLynx™ XS V4.1 SCN803 (Waters Co., Milford, MA, USA). The method parameters were as follows: mass tolerance = 0.05 Da, apex track peak parameters, peak width at 5% height (seconds) = 15/peak-to-peak baseline noise = 50, apply smoothing = yes, collection parameters, intensity threshold (counts) = 100/mass window = 0.05/retention time window = 0.10, noise elimination level = 6, deisotope data = yes. R2 (cumulative) and Q2 (cumulative) were used to determine the validity of the model. R2 (cum) indicates the variation described by all components in the model, and Q2 is a measure of how accurately the model can predict class membership. For the univariate analysis of the unknown compounds, the decreased and increased *m/z* values with a correlation were extracted by estimation of the peak shape and complete separation during the extracted ion monitoring.

2.6. Identification of unknown compounds by databases

The elemental compositions of the unknown compounds on the S-plot and univariate analysis were identified based on the accurate mass and the values of mDa (the difference from the exact mass) and i-FIT (the correctness of isotope patterns of elemental composition; the lower i-FIT normalized values mean high) of each candidate. The possible compounds with the good values of mDa and i-FIT level were extracted from these prospective formulas, and matching the MS spectra of the unknowns to standard model compounds for nicotinic acid and nicotinamide in this study. The MarkerLynx™ XS V4.1 SCN803 combined lists of biomarker candidates were extracted from four metabolomics databases such as NIST (http://www.nist.gov/pml/data/asd.cfm), KEGG (http://www.kegg.com/), ChEBI (http://www.ebi.ac.uk/chebi/) and Food and Agriculture Organization of United Nations (http://faostat.fao.org/site/291/default.aspx). A mass tolerance of 5.0 mDa was set as well as the maximum elemental composition of C = 500, H = 1000, N = 200, O = 200, S = 10, P = 10, and Cl = 10.

3. Results and discussion

3.1. LC/MS assay of untargeted compounds in infant formula

For the evaluation of the untargeted compounds in infant formula, we used LC/MS with positive mode regarding to a chemical fingerprint metabolomics approach. Thus, many low-molecular-weight compounds in the infant formulas are more possibility detected for the exhaustive LC–MS with positive mode than negative mode. We then proposed that the LC/MS assay of the infant formula would be applied for the investigation of the quality and safety assessment. Our previous study showed that LC/MS instrument with various columns on both positive and negative modes dissipated about twice as productive, sample volume and running time as possible. Thus, in the first step, our preliminary study assumed a useful, productive and applicable assay for the quality and safety assessment using fingerprinting metabolomics based on LC/MS with ESI-positive mode. Because there was a reason for relatively large number of m/z values in ESI-positive mode compared to negative mode (Inoue et al., 2015). In this study, the preliminary challenge and analytical procedure was described for the possible food quality and safety of infant formula. Moreover, this aim is that the useful, simple and accurate centrifugal ultrafiltration of low-molecular-weight compounds is utilized for the infant formulas. The centrifugal ultrafiltration is easy to use and has a shorter operating time, resulting in a high recovery and reproducibility of various analytes, thereby saving analytical time and solvents for LC–MS assay (Inoue, Obara, Hino, & Oka, 2010). The smaller size for the cut-off is MW 3000 from the Millipore Corporation. This size can be used for the preparation of non-targeted compounds (MW < 1000) in the infant formulas for our LC/MS assay. Using this preparation of sample, representative LC/MS chromatogram of the untargeted compounds based on ESI-positive scan mode was shown in Fig. 1. The time spent by an individual analyte in each of the two phases (column and mobile phases) is called the capacity factor or retention factor "k". In the RP mode (Fig. 1(A)), many compounds showed the capacity factor $k = 0$ for the LC/MS assay of the untargeted compounds in the infant formulas. The detected numbers in the infant formulas are equal to or lower than 800 from k (>0.5) to 30 min using the gradient Type A mode. On the other hand, in the HILIC mode (Fig. 1(B)), the detected numbers in the infant formulas were between approximately 1100 and 2500 from k (>0.5) to 30 min for the gradient Type B mode. Infant formula is particularly rich in highly polar compounds and diluted by water for making powdered milk.

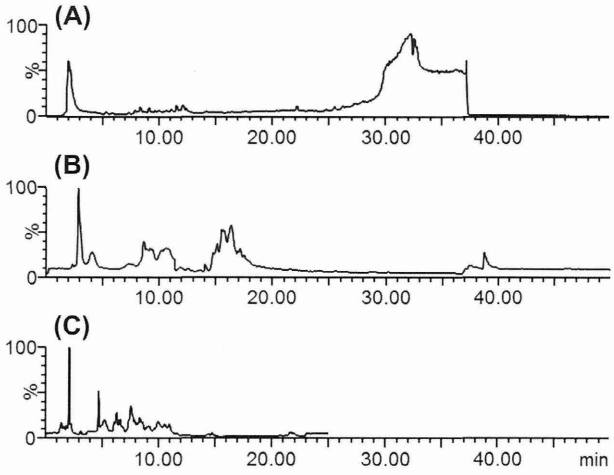

Fig. 1. LC–MS chromatograms of non-targeted compounds in infant formula for comparing the three columns. (A) Column: Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm); gradient mode: Type A; flow rate: 0.2 mL/min; detected numbers: about 800. (B) Column: TSKgel NH_2 column (3 μm, 2.1 × 150 mm); gradient mode: Type B; flow rate: 0.2 mL/min; detected numbers: about 1200. (C) Column: ultra-performance TSKgel Amide-80 column for custom-order (2.0 μm, 2.0 × 150 mm); gradient mode: Type C; flow rate: 0.4 mL/min; detected numbers: about 1200.

The HILIC mode has a more useful and accurate ability to monitor various compounds that have a high to lower polarity in the infant formulas than RP mode. Moreover, in our study for targeting the infant formulas, the metabolomics require a specialized HILIC mode for the LC/MS assay. Recent LC/MS assays used high throughput separation such as ultra-high-pressure and ultra-performance LC techniques (Rogachev & Aharoni, 2012). Thus, the ultra-performance HILIC model column (TSKgel Amide-80, 2 μm) was exceptionally obtained and evaluated for screening the untargeted compounds in the infant formulas (Type C). For this result, our ultra-performance LC/MS assay was performed to detect similar numbers and chromatogram patterns in the normal HILIC mode and to save analytical time for one-run (Fig. 1(C)). The ultra-performance HILIC column could be applied for the untargeted metabolomics that the highest analysis of many possible analytes covering a wide range of hydrophilic property is adopted in a short time.

3.2. Multivariate statistical analysis of infant formula

Good practice could be to perform a preliminarily study to identify the sources of error and/or unwanted delays that could derail a large experimental process. Important issues should also be considered (e.g., industrial process, sampling and preparation) for screening the food quality and safety regarding the unknown compounds in the infant formulas. In this study, the validation was performed using the repeatability of PCA trends from LC/MS data of four infant formulas. PCA can be used to visualize trends in complex data batches ($n = 8$) by a multivariate statistical analysis. These trends of the different batches can be visualized on each point of two components from spectrally-discriminated compounds in the prepared infant formula solutions (Inoue et al., 2015). We tried to find the repeatability of two component's plots which show the distribution behavior of spots from the batches (Supplementary data, Fig. S-1). If the distribution region of each group is significantly expanded, the repeatability of this batch is not good based on the signal intensities and/or retentions of the unknown compounds in the sample group. If the distribution region of each group is exclusively concentrated, the repeatability is good based on the signal intensities of the ions of the unknown compounds in the batch. The repeatability of the signal intensities of the PCA values and detected numbers from the four batches are

Table 1
The repeatability of signal intensities of unknown compounds and PCA values and detected numbers from four samples.

Infant formula	Component 1		Component 2		Detected numbers ± SD
	Averaged value	Absolute RSD (%)	Averaged value	Absolute RSD (%)	
Batch A	41.5	8.2	40.6	12.3	1974 ± 104
Batch B	−43.5	10.0	30.5	13.2	2568 ± 139
Batch C	−47.8	7.5	−32.9	1.9	2547 ± 135
Batch D	49.8	6.2	−38.3	7.7	2073 ± 115 (n = 8)

shown in Table 1. These RSD values for all groups were less than 15% that was satisfactory and used for grouping each infant formula using the LC/MS assay. Due to our approach, this assay are established based on different patterns that showed the chemical fingerprints in steady-state infant formula by rigorous quality and safety assessment. Thus, the chemical profiling and/or fingerprinting of different patterns under steady-state conditions could be demonstrated, and is applied for the various quality assessment of infant formulas by LC/MS assay and PCA plot. In addition, the classification of various kinds of infant formulas was determined by the LC/MS assay and PCA plots (Fig. 2). These results showed that the chemical patterns in the infant formulas could be divided into different and similar types of origin based on the low-molecular-weight compounds. In our study, these untargeted compounds in LC/MS chromatogram would be characterized for the assessment of the unexpected contaminations and degradations by variation in PCA plots.

3.3. Application for the evaluation of contamination of infant formula

In 2008, Chinese milk adulterated with unknown compounds such melamine was a food safety incident concerning the World Health and Food members. At that time, this melamine was difficulty detected regardless of the concentration levels reached as high as 2500 parts per million (ppm), higher than the recorded U.S. levels (Tyan et al., 2009; Xin & Stone, 2008). For future evaluation of unknown and/or unexpected contaminations of low-molecular-weight compounds in infant formula, the metabolomics approach using our LC/MS assay and multivariate statistical analysis is developed to monitor melamine contamination by way of example only. Actually, the melamine contamination (10 and 100 ppm) in the infant formulas was evaluated using LC/MS assay (Fig. 3). These trends of infant formulas in PCA plot can be separated in the melamine contaminations (Fig. 3(A)). In our results, the contamination levels from 10 to 100 ppm could be evaluated by LC/MS assay of unknown obvious peaks at constant contents of infant formula. Moreover, these patterns can be divided on the score plot from the OPLS-DA axis between the control and melamine contamination of the infant formula (Fig. 3(B)). The distinguishing division on the component 1 axis between the control and melamine contamination of the infant formula was observed, and used to calculate the variation in the extremely-different main LC/MS chromatogram pattern (Fig. 3(C)). Indeed, the chemical fingerprinting of the LC/MS chromatogram pattern has been demonstrated, and is opening future possibilities for the food contamination such as mycotoxins, pharmaceuticals and pesticides, in fresh orange juice (Tengstrand, Rosén, Hellenäs, & Aberg, 2013).

3.4. Application of the degradations for the evaluation of markers in infant formula

The time from room to freezing temperatures is very important because on-going food reactions may modify the metabolic contents of the sample. Unexpected accident related to time degradation is a factor that may generate false safety, and that storage-stable infant formula is indicated for keeping the quality control. In this experiment, the storage model of infant formula was configured for one week at room temperature (about 25 °C), 37 °C, 4 °C and freezing (−80 °C) compared to the control (powder storage). The PCA plot and S-plot (Control vs. 37 °C condition) are shown in Fig. 4. The PCA for all samples based on the storage model of the infant formulas revealed an interesting distribution in these groups (Fig. 4(A)). The room temperature and 37 °C results of the groups converged on a particular set in the PCA. These groups, such as room temperature, 37 °C, 4 °C, freezing and the control indicated a disproportionate lack of coherence in the PCA patterns of the detected low-molecular-weight compounds. A feature of the different patterns is their contribution to the PCA plots with integrated low-molecular-weight compounds, which indicates that they are very important markers for fingerprinting the degradation of the infant formulas. Thus, OPLS-DA was used to differentiate between 37 °C and the control groups (Fig. 4(B)). Each point represents the detected low-molecular-weight compounds; the X-axis represents the variable contribution, and the further this pair point

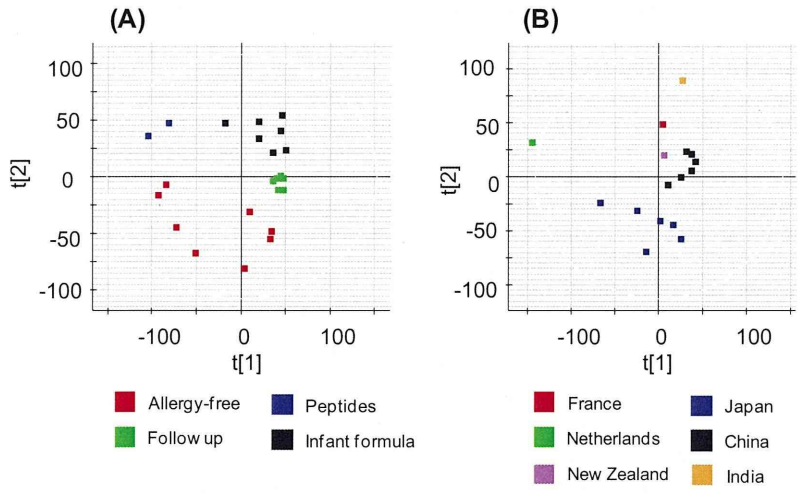

Fig. 2. PCA plots for the classification of various kinds of infant formulas and original countries of origin. (A) PCA plot from four kinds of allergy-free (n = 8), peptides (n = 2), follow up (n = 6) and normal (n = 7) types. (B) PCA plot from 6 countries including France (n = 1), Netherlands (n = 1), New Zealand (n = 1), Japan (n = 6), China (n = 6) and India (n = 1).

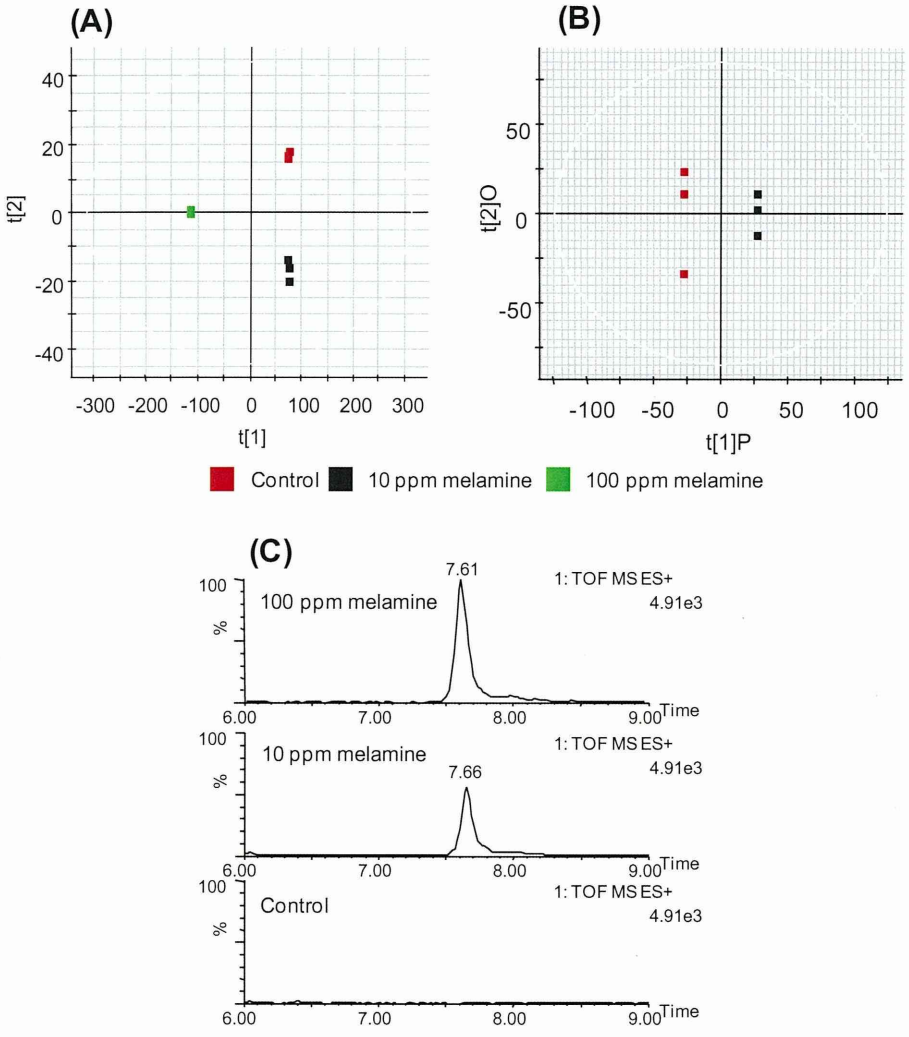

Fig. 3. The trend of PCA plot, score plot from OPLS-DA and typical chromatograms from specific contamination of melamine in infant formula. (A) PCA plot from contamination of melamine in infant formula ($n = 3$). (B) Score plot from OPLS-DA between control and contamination of melamine (10 ppm). (C) Typical extracted chromatograms (m/z 127.073) from control and contamination of melamine (10 and 100 ppm).

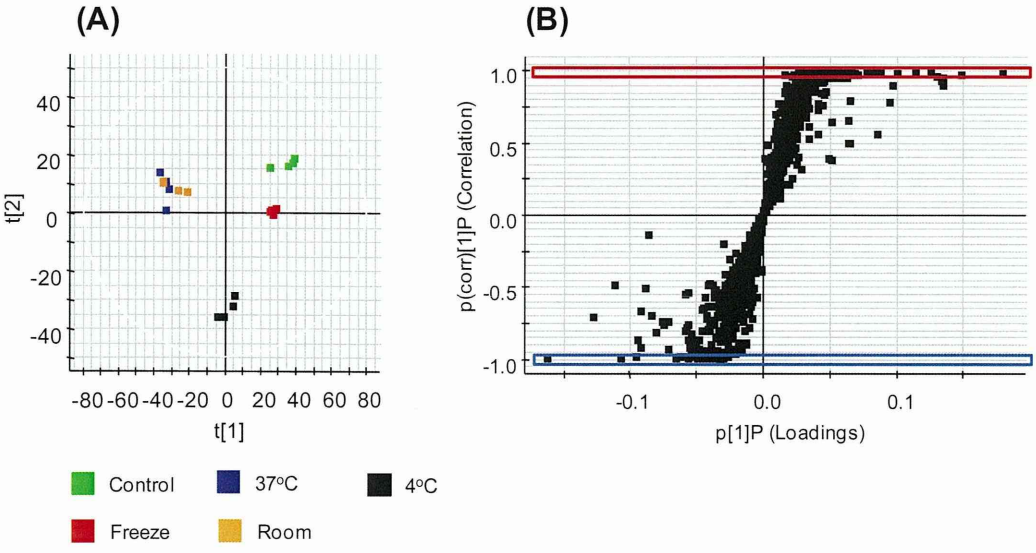

Fig. 4. The trend of PCA plot and S-plot from degradation of infant formula for one week at room temperature (about 25 °C), 37 °C, 4 °C and freezing (−80 °C) compared to control (powder storage). (A) PCA plot from degradation of infant formula for one week ($n = 4$). (B) S-plot between control and 37 °C condition based on OPLS-DA. Increased (red-box: 92 peaks) and decreased (blue-box: 49 peaks) peaks show the possible markers in the degradation of infant formula. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)