要な因子である溶媒特性に着目し詳細な検討を行った。さらに、公定法の有力候補であった WST-1 法にも着目し、各種抗酸化物の活性値を明らかにするとともに、DPPH との活性値の相関性を検討すると共に両者の特性の違いを詳細に検討した。また、WST-1 での活性測定に大きな影響を与えると考えられたキサンチンオキシダーゼ阻害活性を別途検討することにより、WST-1 の有用性についても検討を行った。

B. 研究方法

(1) 対象化合物

抗酸化物（酸化防止剤）として、カテキン水和物、エピカテキン(EC)、エピガロカテキン(EGC)、エピカテキンガレート(ECg)、エピガロカテキンガレート(EGCg)、ケルセチン水和物、ケンフェロール、ケルセチン-3-グルコシド、ルチン、ミリセチン、モリン水和物、ルテオリン、フェルラ酸、ピロカテコール、カフェ酸、没食子酸水和物、セサモール、エラグ酸、アスコルビン酸、グルタチオン（還元型）、α-トコフェロールを用いた。

(2) DPPH ラジカル消去活性測定法

試験管に試料溶液 200 μL、100 mM Tris-HCl buffer（pH7.4） 800 μL、0.20 mM DPPH エタノール溶液 1 mL を添加し、10 秒間激しく攪拌した後、暗所で常温にて 30 分間静置した。30 分後、反応溶液の 517 nm における吸光度(As)を測定した。試料溶液の代わりにエタノールを添加した場合の吸光度をコントロール（Ac）とした。また、DPPH 溶液の代わりにエタノールを添加した場合の吸光度をブランクとした。コントロールの吸光度に対する、試料添加時の吸光度の減少の割合から阻害率（%）を求めた（下式）。

$$\text{阻害率}(\%) = \frac{Ac - As}{Ac} \times 100$$

各試料と標準物質であるトロロックスの IC50 (μmoL/mL)をそれぞれ求め、下式によりトロロックス等価活性 TEAC (μmoL TE/μmoL)を算出した。

$$\text{TEAC} = \frac{\text{トロロックスの} IC_{50}(\mu\text{moL/mL})}{\text{試料の} IC_{50}(\mu\text{moL/mL})}$$

(3) WST-1 法（スーパーオキシド消去能測定）

市販の SOD Assay-Kit WST を使用して測定を行った。96 穴マイクロプレートに試料溶液 20 μL、WST working solution を 200 μ L、Enzyme working solution 20 μ L を順次添加し、プレートシェーカーで室温にて 10 分間撹拌した。その後、マイクロプレートリーダーで 450 nm の吸光度を測定した。試料添加時の吸光度を A_{sanple}、試料溶液のかわりに超純水、あるいは 99.5%エタノールを添加した際の吸光度を A_{blank1}、Enzyme working solution のかわりに Dilution buffer を用いた際の吸光度を A_{blank2}、Enzyme working solution のかわりに Dilution buffer を加え、かつ試料溶液のかわりに超純水、あるいは 99.5%エタノールを添加した際の吸光度を A_{blank3} とし、以下の式から各試料の阻害率 (%) を求めた。各試料の阻害曲線を基に、50%阻害を与える試料濃度 IC_{50} を求めた。WST 還元の 50%阻害を示すサンプル溶液 20 μ L に含まれる SOD を 1 単位 (U) と定義し、スーパーオキシド消去能を SOD 等価活性値 SOSA (U / μmol) として示した。

$$\text{阻害率 } (\%) = \frac{(A_{blank1} - A_{blank3}) - (A_{sanple} - A_{blank2})}{(A_{blank1} - A_{blank3})} \times 100$$

(4) キサンチンオキシダーゼ阻害活性測定法

1.5 m L 容チューブに 12.5 mM リン酸カリウム buffer120 μl、200 μM キサンチン緩衝液 50 μ L、5 mU / m L キサンチンオキシダーゼ酵素緩衝液 20 μ L を混合し、37℃で 5 分間プレインキュベーションを行った。エタノールに溶解した試料 10 μ L を添加し 37℃で 10 分間反応を行った。反応後、3%過塩素酸溶液 25 μ L を加え反応を停止した後、HPLC に供した。なお、HPLC システムは島津製作所 SCL-10AVP、カラムはナカライテスク社製

CO AMOAIL $5C_{18}$-AR-Ⅱ (4.6 mm×250 mm) を用い、0.02 M リン酸を移動相として流速 1.00 m L / min、検出波長 290 nm で分析を行った。試料としてエタノールを加えた時の尿酸のピーク面積値を阻害率 0%とし、試料添加時の尿酸のピーク面積値の減少割合から酵素活性阻害率 (%) を求めた。キサンチンオキシダーゼ阻害活性は、50%阻害を与える試料濃度 IC_{50} として示した。

C. 研究結果及び考察

23、24 年度の研究において、DPPH と ABTS 法では、分子内にカテコール構造を有する化合物において活性値に大きな差が生じることを明らかにしている。すなわち、カテキン、EC、ECg、ケルセチン、ケルセチン-3-グルコシド、ルチン、ルテオリン、ピロカテコール、カフェ酸、エラグ酸の計 10 化合物では、活性値が DPPH＞ABTS となり、TEAC 値でおよそ 1.3（μmolTE／μmol）DPPH 法での活性値が高い結果となっている。この原因について検討した結果、DPPH 法では、カテコール構造を有する化合物が、DPPH ラジカル消去後にキノン構造に変化後、溶媒であるエタノールの求核攻撃の影響により再生され、再度活性を発現するものと推察してきた。本年度は、この DPPH 法による溶媒効果を明らかにすることを目的としてアセトニトリル系での活性値を測定した。すなわち、アセトニトリル系での DPPH 測定においてはカテコール構造の再生は起こらないものと考えられていることから、水／エタノール系とアセトニトリル系の活性値を比較検証した（表 1）。対象化合物は昨年度と同様の 21 種の抗酸化物であったが、アスコルビン酸およびグルタチオンはアセトニトリルに不要のため測定不能であった。両者の測定値を比較した結果、カテキン、EC、ECg、ケルセチン、ケルセチン-3-グルコシド、ルチン、ルテオリン、ピロカテコール、カフェ酸、エラグ酸の計 10 種のカテコール化合物では TEAC 値で 1.06～2.20 (μmol-TE/μmol)の活性値の差が認められた。すなわち、アセトニトリル系ではカテコールの再生反応の抑制に起因すると考えられる活性値の低下が顕著に現れる結果となった。また、EGC、EGCg、ミリセチン、没食子酸の活性値も約 1 (μmol-TE/μmol)程度の活性値の低下が認められ、DPPH 法ではカテコール以外にもピロガロール構造の再生も重要な因子となることが示唆された。これらの結果から、DPPH 法での反応系における溶媒の役割と各種抗酸化物の反応特性が明らかとなり、抗酸化物のカテコール・ピロガロール構造の重要性をあらためて確認することができた。

続いて、DPPH 法と WST-1 法の比較検証を試みた。WST-1 法は、キサンチンオキシダーゼ酵素反応を利用したスーパーオキシド消去反応の測定法である。一電子転移反応に基づく化学反応を利用する DPPH 法とは本質的に異なる測定法であるが、両者の活性値の高低に関しては類似点も認められることから、本実験では上記と同様に 21 種抗酸化物の測定を行い、両者の活性値の相違点を詳細に検討した。WST-1 法での測定結果を DPPH 法での活性値と併せて表 2 に示した。なお、WST-1 法では、溶解性の問題から疎水性化合物であるα-トコフェロールの測定が困難であり、グルタチオン、セサモールも活性値が認められなかった。結果として得られた 18 化合物について、DPPH 法と WST-1 法の活性相関を検討し図 1 に示した。その結果、両者の相関係数は 0.660 となり、高い相関は認められなかった。しかしながら、化合物毎に詳細に見てみると、ピロガロール構造を有する EGCg、EGC、ECg、没食子酸は両法で高い活性値を示し、カテコール構造を有する化合物がそれに続く高い活性値を示す傾向が認められ、両法で類似した活性特性が認められた。続いて、キサンチンオキシダーゼの阻害活性評価結果(IC_{50} 値）を表 3 に示した。表に示したように、21 化合物中 10 化合物で阻害活性が認められたが、特にケンフェロール、ケルセチン、ミリセチン、ルテオリンの 4 化合物では IC_{50} 値が 1 μM 以下と極めて阻害性が高い結果となった。WST-1 法ではキサンチンオキシダーゼの作用が引き金となってスーパーオキシドが生じる反応を利用するため、キサンチンオキ

シダーゼの阻害は結果としてWST-1試薬の発色を低下させ、見かけ上活性値を増大させるものと推察していた。しかしながら、高いキサンチンオキシダーゼ阻害活性を示したケルセチンなどは、本WST-1法では期待されるよりも総じて低い活性値を示した。この結果は、本WST-1法ではキサンチンオキシダーゼの阻害効果が必ずしも活性値の見かけ上の増加につながらず、逆に活性値の低下を引き起こす要因になっており可能性も示唆された。しかしながら、WST-1法での反応条件とキサンチンオキシダーゼ阻害試験の反応条件は必ずしも一致していないことから、この点については今後詳細に検討する必要があるものと考えられた。

D. 結論

DPPH法を酸化防止剤の規格試験法として確立するために、各種抗酸化物の反応特性に及ぼす溶媒効果を検証した結果、水／エタノール溶媒中ではカテコール・ピロガロール構造の再生反応が生じ、活性値が増加する傾向になることが確認された。続いて、DPPH法とWST-1法の活性相関を検討した結果、両者の相関は高くないものの類似した活性傾向を示すことが明らかになった。このことは、DPPH法での活性評価にスーパーオキシド消去能もある程度反映されていることを示唆している。以上の結果は、今後酸化防止剤規格試験法の確立に重要な情報となると考えられた。

E. 研究発表

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表１　DPPH 法における水／エタノール系とアセトニトリル系での活性比較

測定化合物	水／エタノール TEAC（μmol TE/μmol）	アセトニトリル TEAC（μmol TE/μmol）	TEAC値の差
カテキン	2.47±0.09	0.48±0.01	1.99
エピカテキン(EC)	2.73±0.08	0.53±0.01	2.20
エピガロカテキン(EGC)	3.12±0.05	2.24±0.04	0.88
エピカテキンガレート(ECG)	4.86±0.21	2.97±0.36	1.89
エピガロカテキンガレート(EGCG)	4.83±0.18	3.72±0.06	1.11
ケンフェロール	1.01±0.01	0.34±0.00	0.67
ケルセチン	3.18±0.03	1.28±0.04	1.90
ミリセチン	3.09±0.01	1.95±0.02	1.14
モリン	0.97±0.01	0.76±0.01	0.21
ケルセチン-3-グルコシド	2.22±0.11	0.99±0.03	1.32
ルチン	2.40±0.11	0.99±0.01	1.41
ルテオリン	2.22±0.07	1.16±0.02	1.06
フェルラ酸	0.71±0.04	0.25±0.00	0.46
ピロカテコール	2.73±0.09	0.81±0.03	1.92
カフェ酸	2.46±0.01	1.03±0.03	1.43
没食子酸	2.52±0.06	1.57±0.05	0.95
セサモール	0.78±0.02	0.29±0.01	0.49
エラグ酸	3.84±0.30	2.02±0.03	1.82
アスコルビン酸	0.87±0.00	測定不能	
グルタチオン(還元型)	0.70±0.00	測定不能	
α-トコフェロール	0.90±0.05	1.01±0.01	-0.11

表２　DPPH 法と WST-1 法での活性値の比較

測定化合物	DPPH (μmol TE/μmol)	WST-1 (U/μmol)
カテキン	2.47±0.09	3066.3±279.7
エピカテキン(EC)	2.73±0.08	2868.5±364.2
エピガロカテキン(EGC)	3.12±0.05	5579.5±641.8
エピカテキンガレート(ECG)	4.86±0.21	4922.8±362.8
エピガロカテキンガレート(EGCG)	4.83±0.18	6951.7±30.5
ケンフェロール	1.01±0.01	1064.6±6.4
ケルセチン	3.18±0.03	489.2±68.5
ミリセチン	3.09±0.11	1351.5±148.9
モリン	0.97±0.01	75.7±0.2
ケルセチン-3-グルコシド	2.22±0.11	670.6±80.0
ルチン	2.40±0.11	374.4±40.8
ルテオリン	2.22±0.07	1462.1±141.6
フェルラ酸	0.71±0.04	117.2±4.3
ピロカテコール	2.73±0.09	1212.3±115.7
カフェ酸	2.46±0.01	4814.9±300.8
没食子酸	2.52±0.06	5505.3±656.9
セサモール	0.78±0.02	n.d.
エラグ酸	3.84±0.30	1947.8±128.4
アスコルビン酸	0.87±0.00	52.0±1.6
グルタチオン(還元型)	0.70±0.00	n.d.
α-トコフェロール	0.90±0.05	n.d.

図１　DPPH 法と WST-1 法の活性相関

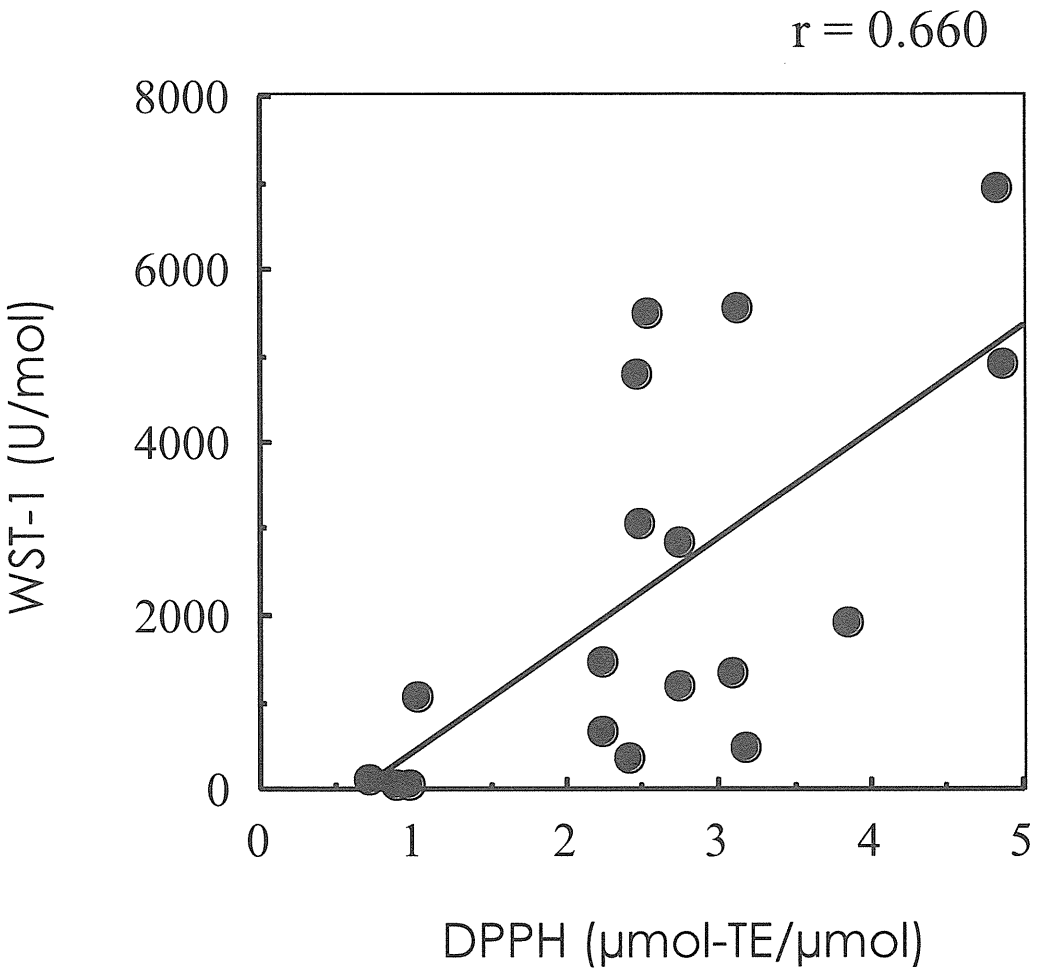

表３　各種抗酸化物のキサンチンオキシダーゼ阻害活性

測定化合物	XO阻害活性 IC_{50} (μM)
カテキン	n.d.
エピカテキン(EC)	n.d.
エピガロカテキン(EGC)	n.d.
エピカテキンガレート(ECG)	14.89±0.50
エピガロカテキンガレート(EGCG)	15.60±0.17
ケンフェロール	0.15±0.00
ケルセチン	0.17±0.03
ミリセチン	0.43±0.01
モリン	4.28±0.04
ケルセチン-3-グルコシド	33.95±0.00
ルチン	30.03±1.23
ルテオリン	0.15±0.00
フェルラ酸	n.d.
ピロカテコール	n.d.
カフェ酸	n.d.
没食子酸	n.d.
セサモール	n.d.
エラグ酸	2.50±0.05
アスコルビン酸	n.d.
グルタチオン(還元型)	n.d.
α-トコフェロール	n.d.

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Scientific Research

Improvement of the Assay Method for Steviol Glycosides in the JECFA Specifications

Atsuko Tada[1*], Kyoko Ishizuki[1], Junichi Iwamura[2], Hirohisa Mikami[3], Yoshiko Hirao[3], Isao Fujita[4], Takeshi Yamazaki[1], Hiroshi Akiyama[1], Yoko Kawamura[1]

[1]National Institute of Health Sciences, Tokyo, Japan
[2]Laboratory of Creative Science Co., Ltd., Misonocho, Japan
[3]Shimadzu Corporation, Kyoto, Japan
[4]Morita Kagaku Kogyo Co., Ltd., Osaka, Japan
Email: *atada@nihs.go.jp

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ABSTRACT

Steviol glycosides are natural sweetener constituents found in the leaves of *Stevia rebaudiana* Bertoni (Asteraceae). The specifications for steviol glycosides were established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, although there was a call in the following year for the modification of this assay method to enable the determination of nine steviol glycosides rather than just seven. In response, based on a proposed method by the Japan Stevia Association, we developed an improved method by changing the HPLC conditions and including the use of an octadecylsilyl column instead of an amino-bonded column to enable the rapid and reliable determination of the nine steviol glycosides by an isocratic HPLC-UV method. With the developed method, the nine steviol glycosides can be separately determined, and identified using individual reference chemicals as standards, unlike the previous identification method, which was based on the relative retention times. In addition, the single stevioside quantification standard was replaced with both stevioside and rebaudioside A quantification standards. Importantly, the validation of the developed method was successful. The limits of quantification for the nine steviol glycosides were between 0.2% and 0.6%. The developed assay method for the nine steviol glycosides was proposed to JECFA and adopted as the revised assay method for the steviol glycosides specifications at its 73rd meeting in 2010.

Keywords: Steviol Glycosides; Stevioside; Rebaudioside A; Reversed-Phase HPLC; JECFA Specifications

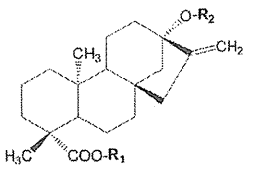
1. Introduction

Steviol glycosides are natural sweeteners found in the leaves of *Stevia rebaudiana* Bertoni (Asteraceae), and their principal components are stevioside and/or rebaudioside A. Stevia extract was accepted as an existing food additive by the Ministry of Health, Labor and Welfare in Japan (1996), and it has been used in many types of Japanese foods. The specifications for "steviol glycosides" were established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) in 2008 [1], and stevia extracts were also approved as generally recognized as safe by the US Food and Drug Administration that year. Since then, purified stevia extracts have been used throughout the world.

In the specifications established by JECFA in 2008 [1], the assay determined the concentrations of seven steviol glycosides: stevioside, rebaudioside A, rebaudioside B, rebaudioside C, dulcoside A, rubusoside, and steviolbioside (**Figure 1**). These compounds were analyzed by HPLC using an amino-bonded column and identified by the zone method with relative retention times to that of rebaudioside A.

In 2009, JECFA required that additional information for the detection of the steviol glycosides rebaudioside D and rebaudioside F (**Figure 1**) included in the assay method. In response to this request, the Japan Stevia Association (JSA) examined the existing test method and proposed a new one, which was then reviewed and improved by the National Institute of Health Sciences (NIHS), and the developed method was reproposed. With this new method, the nine steviol glycosides could be separately analyzed by HPLC using an octadecylsilyl (ODS) column. Moreover, each steviol glycoside was identified by an individual reference chemical rather than by the zone method,

*Corresponding author.



Compound	R_1	R_2	MW
Stevioside	Glc(β)-	Glc(β1-2)Glc(β)-	804.9
Rebaudioside A	Glc(β)-	Glc(β1-2)Glc(β)- Glc(β1-3) ┘	967.0
Rebaudioside B	H-	Glc(β1-2)Glc(β)- Glc(β1-3) ┘	804.9
Rebaudioside C	Glc(β)-	Rha(α1-2)Glc(β)- Glc(β1-3) ┘	951.0
Rebaudioside D	Glc(β1-2)Glc(β)-	Glc(β1-2)Glc(β)- Glc(β1-3) ┘	1129.2
Rebaudioside F	Glc(β)-	Xyl(β1-2)Glc(β)- Glc(β1-3) ┘	937.0
Steviolbioside	H-	Glc(β1-2)Glc(β)-	642.7
Dulcoside A	Glc(β)-	Rha(α1-2)Glc(β)-	788.9
Rubusoside	Glc(β)-	Glc(β)-	642.7

Glc: D-glucose, Rha: L-rhamnose, Xyl: D-xylose

Figure 1. Structures and molecular weights of steviol glycosides.

and the quantification was achieved using both stevioside and rebaudioside A standards. Thus, this analysis provided higher accuracy and reliability. Subsequently, this method was adopted as the revised assay method for steviol glycosides at the 73rd JECFA meeting in 2010 [2]. This paper describes the basis of the new method developed by both JSA and NIHS.

2. Experimental

2.1. Reagents and Samples

The standards for stevioside (99.0+% (HPLC, dried), Code No. 193-15351) and rebaudioside A (99.0+% (HPLC, dried), Code No. 183-02361) used for the quantification were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). The other seven steviol glycosides (rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside, and steviolbioside) were prepared from stevia extracts by the Laboratory of Creative Science Co., Ltd. (Osaka, Japan). The purity of rebaudioside F was approximately 20%, while that of the other compounds was greater than 90%. A reference standard mixture containing the nine steviol glycosides for peak identification was prepared by dissolving nine standards in a 30:70 (v/v) mixture of acetonitrile and water, which are currently marketed by Wako Pure Chemical Industries. Two samples of commercial stevia extracts used as food additives in Japan, RA95 and RA60, were obtained from Morita Kagaku Kogyo Co., Ltd., Japan.

2.2. HPLC

The analysis was performed using an Alliance 2695 HPLC system (Waters Co., MA, USA) with an SPD-10AV UV-Vis detector (Shimadzu Corporation, Kyoto, Japan). Separation was carried out on 4.6 × 250 mm ODS columns with a particle size of 5 μm: Capcell Pak C18 MGII (Shiseido Co., Ltd., Tokyo, Japan), Shim-pack CLC-ODS (Shimadzu Corporation), and Luna C18 (Phenomenex Co., Ltd., CA, USA). For comparison, a Sun-Fire C18 column (Waters Co., Ltd.) and other ODS columns were also used. The column temperature was maintained at 40°C. Elution was achieved using a 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) as the mobile phase for 30 min. The flow rate was maintained at 1.0 mL/min, and the chromatographic profile was monitored at 210 nm.

For comparison, the existing JECFA method was performed using a Supelcosil LC-NH2 amino-bonded column (4.6 mm i.d. × 250 mm, 5 μm, Sigma-Aldrich Co., MO, USA). The column temperature was maintained at 40°C. Elution was achieved at a flow rate of 1.0 mL/min using an 80:20 (v/v) mixture of acetonitrile and water adjusted to a pH of 3.0 with phosphoric acid as the mobile phase, and the chromatographic profile was monitored at 210 nm.

2.3. Test Method

For the standard solutions, each of the stevioside and rebaudioside A standards (50 mg each after drying at 105°C for 2 h) was accurately weighed into separate 50 mL volumetric flasks, and the volume was made up with a 30:70 (v/v) mixture of acetonitrile and water. They were diluted as appropriate with the same solvent mixture. For the sample solutions, RA95 and RA60 (50 mg each after drying at 105°C for 2 h) were accurately weighed into separate 50 mL volumetric flasks, and the volume was made up with a 30:70 (v/v) mixture of acetonitrile and water. The standard and sample solutions (5 μL) were injected into the HPLC system. Steviol glycosides

were identified on the basis of their correspondence of retention times using the reference standard mixture, and the peak areas were measured. Each solution was injected in triplicate, and the mean value was used for quantitation.

2.4. Calculation of Steviol Glycoside Concentration

The concentration of eight (not rebaudioside A) steviol glycosides (x) in the sample was calculated by the following formula:

$$Cx(\%) = \frac{W_{stv-stn} \times f_x \times A_{x-smp} \times 100}{W_{smp} \times A_{stv-stn}} \quad (1)$$

Here Cx (%) is the concentration of a steviol glycoside, $W_{stv-stn}$ is the weight of the stevioside standard (dried basis), W_{smp} is the weight of the sample (dried basis), A_{x-smp} is the peak area of the steviol glycoside (x) in the sample solution, $A_{stv-stn}$ is the peak area of the stevioside in the standard solution, and fx is the coefficient value for each steviol glycoside (1.00 (rebaudioside B), 1.18 (rebaudioside C), 1.40 (rebaudioside D), 1.16 (rebaudioside F), 0.98 (dulcoside A), 0.80 (rubusoside), or 0.80 (steviolbioside)).

The concentration of rebaudioside A in the sample was calculated by the following formula:

$$C_{reA(\%)} = \frac{W_{reA-stn} \times A_{reA-smp} \times 100}{W_{smp} \times A_{reA-stn}} \quad (2)$$

Here C_{reA} (%) is the concentration of rebaudioside A, $W_{reA-stn}$ is the weight of the rebaudioside A standard (dried basis), W_{smp} is the weight of the sample (dried basis), $A_{reA-smp}$ is the peak area of rebaudioside A in the sample solution, and $A_{reA-stn}$ is the peak area of rebaudioside A in the standard solution.

The concentration of total steviol glycosides was then calculated from the sum of the nine steviol glycoside concentrations.

3. Results and Discussion

3.1. Analysis Using the Existing JECFA Method

In the JECFA method established in 2008 [1], each steviol glycoside was identified by the relative retention time to that of rebaudioside A. **Figure 2** shows the HPLC chromatogram of the nine steviol glycosides obtained according to the method using the amino-bonded column.

As can be seen in **Figure 2**, newly added rebaudioside D appears at 40 min with a broad pattern and low sensitivity, although the other steviol glycosides are eluted within 20 min. In addition, although we performed the analysis of the nine steviol glycosides using the identical amino-bonded column described in the original JECFA method, the peaks of rubusoside and steviolbioside were not fully separated, as shown in **Figure 2**. Moreover, some retention times of the peaks of the steviol glycolsides did not correspond with the relative retention times to that of rebaudioside A as described in the original JECFA method. We then attempted to separate the nine steviol glycosides using four other amino-bonded columns—Supelcosil LC-NH2-NP (4.6 mm i.d. × 250 mm, 5 μm, Sigma-Aldrich Co.), Asahipak NH2P-50 4E amino-bonded column (4.6 mm i.d. × 250 mm, 5 μm, Showa Denko Co., Ltd., Kawasaki, Japan), TSKgel NH2-100 (4.6 × 150 mm, 3 μm, Tosoh Co., Tokyo, Japan), and Wakosil 5NH2 (4.6 × 250 mm, 5 μm, Wako Pure Chemical Industries Co., Ltd.). Again, similar problems were observed (data not shown). Although the reason for the insufficient separation is unclear, the instability of ami no-bonded columns is one of the problems with the conventional method. The amino-bonded columns have a tendency to rapidly deteriorate. These results suggested that another type of column should be introduced. Therefore, we attempted to develop an analytical method to solve these problems.

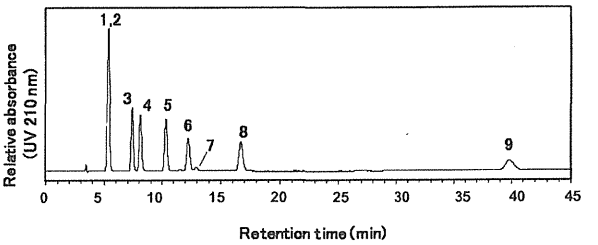


Figure 2. HPLC chromatogram of the reference standard mixture of nine steviol glycosides using an amino column as described in the conventional JECFA method. Separation was carried out on an supelcosil LC-NH2 column (4.6 mm i.d. × 250 mm, 5 μm) at a column temperature of 40°C using an 80:20 (v/v) mixture of acetonitrile and water adjusted to a pH of 3.0 with phosphoric acid as the mobile phase at a flow rate of 1.0 mL/min. the eluted compounds were monitored at 210 nm. 1: rubusoside, 2: steviolbioside, 3: dulcoside A, 4: rebaudioside B, 5: stevioside, 6: rebaudioside C, 7: rebaudioside F, 8: rebaudioside A, 9: rebaudioside D.

3.2. HPLC Using ODS Columns

To solve these problems, the use of an ODS column was attempted for the determination of the nine steviol glycosides. With respect to the organic solvent for the mobile phase, acetonitrile was found to give a better separation than methanol (data not shown). Next, to determine the appropriate pH of the mobile phase, six mobile phases based on 32:68 (v/v) mixtures of acetonitrile and acidic solutions, including 10 mmol/L sodium phosphate buffer (pH 2.6), 1.4 mmol/L phosphoric acid (pH 3.0), 5 mmol/L formic acid (pH 3.0), 5 mmol/L ammonium formate (pH 6.1), and 5 mmol/L ammonium acetate (pH 6.6), and H_2O were used.

As shown in **Figure 3**, the nine steviol glycosides were separately detected within 30 min using five of the mobile phases under isocratic conditions. Only the mobile phase containing 5 mmol/L ammonium acetate (pH 6.6) was not effective (**Figure 3(e)**). Rebaudioside B and steviolbioside appear to elute faster using higher pH mobile phases. The mobile phase formulated with sodium phosphate buffer (10 mmol/L, pH 2.6) was found to provide the best peak shape and sensitivity (**Figure 3(a)**).

Next, the ratio of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) in the mobile phase was varied (35:65, 32:68, and 30:70). As can be seen in **Figure 4(a)**, when the 35:65 (v/v) mixture of acetonitrile and sodium phosphate buffer was used as the mobile phase, the retention time of rebaudioside D was very close to the peak signal caused by injection shock. In the case of the 30:70 (v/v) mixture, rebaudioside B and steviolbioside were eluted after more than 30 min with broad peaks (**Figure 4(c)**). Both of the peaks of rebaudioside D and the injection shock were identically detected using other ODS columns (data not shown). Fortunately, with the 32:68 (v/v) mixture of acetonitrile and sodium phosphate buffer, the injection shock and rebaudioside D peaks were separated, and the rebaudioside B and steviolbioside peaks had a good shape and retention time. Based on these results, the 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) was selected as the mobile phase. Under the analytical conditions, the UV spectra of the steviol glycosides showed the strongest UV absorption near 200 - 210 nm (data not shown).

To confirm the equality of different ODS columns, the analysis was carried out using more than six commercial ODS columns, and the results were compared. As can be seen in **Figure 5**, the elution patterns of the nine steviol glycosides were equivalent among four of the ODS columns, and the resolution of the rebaudioside A and stevioside peaks for the Capcell Pak C18 MGII (a), Shimpack CLC-ODS (b), Luna C18(2) (c), and SunFire C18 (d) columns was 1.65, 1.60, 1.55, and 1.42, respectively. However, stevioside and rebaudioside A could not be separated on some of the ODS columns (**Figure 5(e)**). Therefore, ODS columns with the appropriate level of resolution should be selected for the analysis of the nine steviol glycosides.

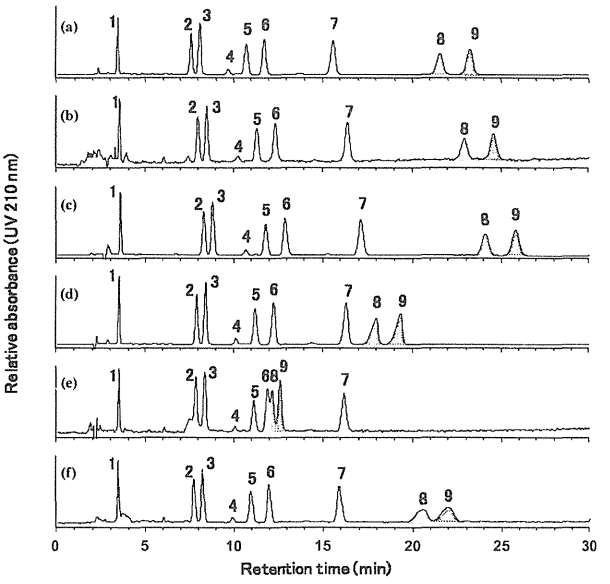


Figure 3. HPLC chromatograms of the reference standard mixtures of nine steviol glycosides obtained using an ODS column with six different mobile phases. the mobile phases were 32:68 (v/v) mixtures of acetonitrile and various solutions, including (a) 10 mmol/L sodium phosphate buffer (pH 2.6), (b) 1.4 mmol/L phosphoric acid (pH 3.0), (c) 5 mmol/L formic acid (pH 3.0), (d) 5 mmol/L ammonium formate (pH 6.1), (e) 5 mmol/L ammonium acetate (pH 6.6), and (f) H_2O. Separation was carried out on a capcell pak C18 MGII column (4.6 mm i.d. × 250 mm, 5 μm) at a column temperature of 40°C and a flow rate of 1.0 mL/min and was monitored at 210 nm. 1: rebaudioside D, 2: rebaudioside A, 3: stevioside, 4: rebaudioside F, 5: rebaudioside C, 6: dulcoside A, 7: rubusoside, 8: rebaudioside B, 9: steviolbioside.

3.3. Calibration Curves for the Developed Assay Method

The calibration curves were constructed for a range of

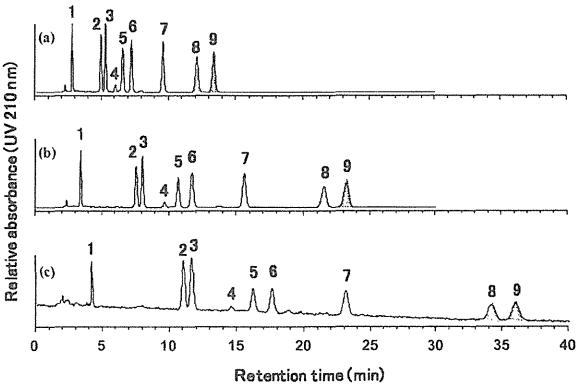


Figure 4. HPLC chromatograms of the reference standard mixture of nine steviol glycosides using different mobile phases. Mobile phases were 35:65 (v/v) mixtures of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) (a), 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) (b) or 30:70 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) (c). Separation was carried out on capcell pak C18 MGII column (4.6 mm i.d. × 250 mm, 5 μm) at a column temperature of 40°C and a flow rate of 1.0 mL/min. The eluted compounds were monitored at 210 nm. 1: rebaudioside D, 2: rebaudioside A, 3: stevioside, 4: rebaudioside F, 5: rebaudioside C, 6: dulcoside A, 7: rubusoside, 8: rebaudioside B, 9: steviolbioside.

nine concentrations of the stevioside and rebaudioside A standards (**Figure 6**). Good linearity was achieved over the concentration range from 0.001 to 2.0 mg/mL for both the stevioside and rebaudioside A standards. The correlation coefficients (R^2) for the two steviol glycolsides were more than 0.9999. The limits of quantification (LOQ) (S/N = 10) for stevioside and rebaudioside A were 3 μg/mL each in the standard solutions and 0.3% each in the sample mixture. Good linearity was also achieved for the other seven steviol glycosides (data not shown) over the concentration range from approximately 0.001 to 0.5 mg/mL for rebaudioside D, rebaudioside C, dulcoside A, rubusoside, rebaudioside B, and steviolbioside, and 0.0001 - 0.1 mg/mL for rebaudioside F. The correlation coefficients for the seven steviol glycosides were more than 0.999. LOQ (S/N = 10) for the seven steviol glycosides—rebaudioside D, rebaudioside F, rebaudioside C, dulucoside A, rubusoside, rebaudioside B and steviolbioside—were 2, 4, 4, 4, 4, 6, and 5 μg/mL, respectively, in the standard solutions and 0.2%, 0.4%, 0.4%, 0.4%, 0.4%, 0.6%, and 0.5%, respectively, in the sample mixture. Therefore, the linearity and sensitivity of the method are acceptable.

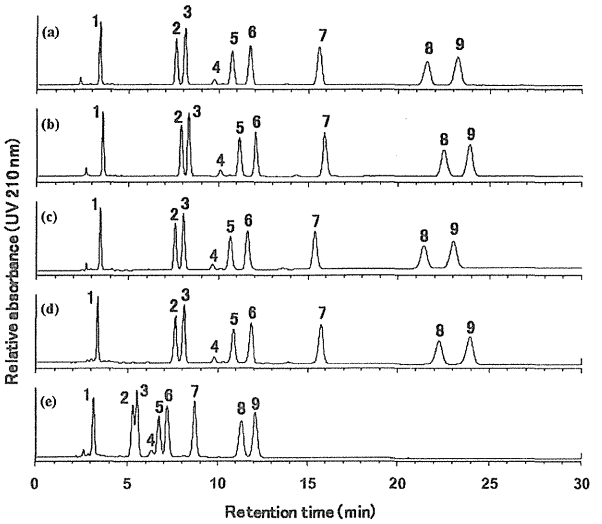


Figure 5. HPLC chromatograms of the reference standard mixture of nine steviol glycosides using different ODS columns (4.6 × 250 mm, 5 μm), Capcell Pak C18 MGII (a), Shim-pack CLC-ODS (b), Luna C18(2) (c), SunFire C18 (d), or other ODS column (e). Mobile phase was 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer. Separation was carried out at a column temperature of 40°C at the flow rate of 1.0 mL/min. The eluted compounds were monitored at 210 nm. 1: rebaudioside D, 2: rebaudioside A, 3: stevioside, 4: rebaudioside F, 5: rebaudioside C, 6: dulcoside A, 7: rubusoside, 8: rebaudioside B, 9: steviolbioside.

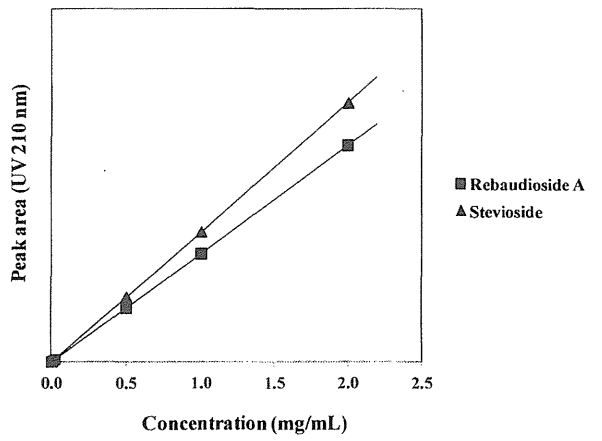


Figure 6. Calibration curves for the stevioside and rebaudioside A standards.

3.4. Verification of the Developed Assay Method

As shown in **Table 1**, the precision of the method was tested using multiple injections of high-purity samples of stevioside and rebaudioside A (n = 3). The relative standard deviation (RSD) for the amount of stevioside and rebaudioside A determined in the samples was calculated to be 0.41% and 0.70%, respectively. These RSD values are acceptable.

3.5. Application of the Developed Method to Commercial Stevia Extracts

To ensure the applicability of the developed method, the quantities of the nine steviol glycosides in two types of commercial stevia extracts, RA 95 and RA 60, were determined. The reference standard mixture of the nine steviol glycosides was used for the identification of the peaks. As shown in **Figure 7**, both samples contained mainly rebaudioside A, although RA 60 also contained some levels of stevioside and rebaudioside C.

The quantification results are summarized in **Table 2**. The values were calculated using the rebaudioside A standard for rebaudioside A and the stevioside standard for the other eight steviol glycosides. The percentage of total steviol glycosides in the two stevia extracts, RA95 and RA60, was 99.5% and 88.9%, respectively.

Table 3 shows the quantified values of rebaudioside A and the total value of the steviol glycosides in the stevia extracts calculated using the rebaudioside A standard and the stevioside standard. Rebaudioside A was analyzed by the developed HPLC method, and then the levels of rebaudioside A in the stevia extracts, RA 95 and RA60, were determined by the two different calculation methods with the stevioside standard or the rebaudioside A standard. In the previous JECFA method, the rebaudioside A levels were determined using the stevioside standard as f_x = 1.20 in Equation (1). However, in the developed method in this study, an actual rebaudioside A

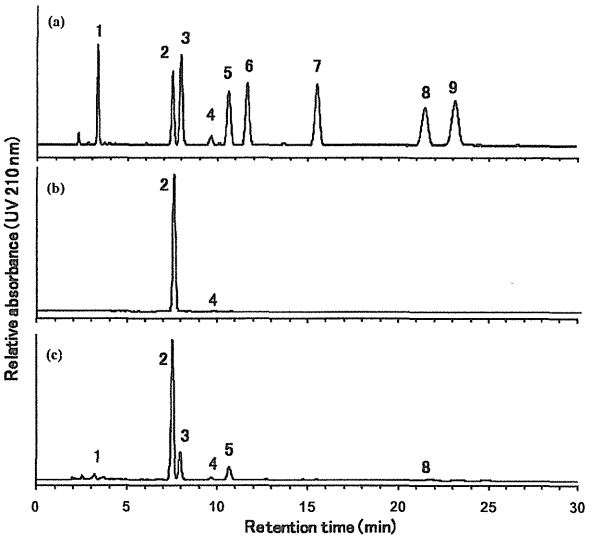


Figure 7. HPLC chromatograms of the reference standard mixture of nine steviol glycosides (a), stevia extracts used as food additives, RA 95 (b) and RA 60 (c). Separation was carried out on a Capcell Pak C18 MGII column (4.6 mm × 250 mm, 5 μm) at a column temperature at 40°C and a flow rate of 1.0 mL/min. The eluted compounds were monitored at 210 nm. Mobile phase was 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer. 1: rebaudioside D, 2: rebaudioside A, 3: stevioside, 4: rebaudioside F, 5: rebaudioside C, 6: dulcoside A, 7: rubusoside, 8: rebaudioside B, 9: steviolbioside.

Table 1. Quantification of stevioside and rebaudioside A in their high-purity samples by the developed HPLC method.

Sample	Content (%)				
	n = 1	n = 2	n = 3	Mean	RSD (%)
Stevioside	99.8	100.5	100.4	100.4	0.41
Rebaudioside A	99.5	99.6	100.7	99.9	0.70

standard was adopted for the determination of rebaudioside A, because high levels of rebaudioside A have recently been detected in many stevia extracts, and high-purity rebaudioside A standards have become commercially available. As shown in the table, the levels of rebaudioside A and total steviol glycosides in both RA95 and RA60 determined by the developed calculation method appear to be very slightly lower than those obtained by the previous calculation method. The levels obtained by the newly developed method are not significantly different from those obtained by the previous method, and both levels are within the range of the measurement error (0.73%). Scientifically, in an HPLC quantification method, the target compound should be quantified by a standard curve based on a standard of the identical compound. The levels of rebaudioside A determined using the rebaudiosideA standard are more precise and reliable than

Table 2. Quantification of the nine steviol glycosides in stevia extracts used as food additives using HPLC.

Steviol glycoside	Content (%)[a]			
	RA95		RA60	
	Mean	±SD	Mean	±SD
Rebaudioside D	0.24	0.01	2.09	0.08
Rebaudioside A[b]	98.7	0.73	65.3	0.73
Stevioside	ND		10.7	0.08
Rebaudioside F	0.52	0.00	1.35	0.01
Rebaudioside C	ND		7.56	0.09
Dulcoside A	ND		ND	
Rebaudioside B	ND		1.41	0.03
Rubusoide	ND		0.45	0.01
Steviolbioside	ND		ND	
Total steviol glycoside	99.5	0.73	88.9	0.99

[a]On the dried basis. [b]Rebaudioside A was quantified by rebaudioside A standard. [c]ND: Not detected. Each value is a mean of three trials.

Table 3. Comparison of the rebaudioside A concentration in stevia extracts using the two different calculation methods.

	Content (%)[a]			
	Previous calculation method[b]		Developed calculation method[c]	
	RA95	RA60	RA95	RA60
Rebaudioside A	99.1	65.6	98.7	65.3
Total steviol glycoside	99.8	89.2	99.6	88.9

[a]On the dried basis. [b]Rebaudioside A was quantified by stevioside standard. [c]Rebaudioside A was quantified by rebaudioside A standard.

those obtained using a standard that is a different compound and requires coefficient value, particularly for stevia extracts with high concentrations of rebaudioside A. Therefore the rebaudioside A standard was used for the quantification of rebaudioside A in the developed method.

4. Conclusion

We developed an isocratic HPLC-UV method by employing an ODS column using a reference standard mixture of nine steviol glycosides. On the ODS column, the steviol glycosides can be detected separately and sensitively within 30 min. In addition, the use of the reference standard mixture of nine steviol glycosides enables the correct identification of the steviol glycosides in different samples. The reference standard mixture of the nine ste-

viol glycosides is now commercially available. The rapid and reliable determination of the nine steviol glycosides by an isocratic HPLC-UV method on an ODS column was first developed in this study and has not been previously reported [3-7]. Subsequently, this method was proposed as the revised assay method for steviol glycosides and adopted at the 73rd JECFA meeting in 2010 [2] .

5. Acknowledgements

We are grateful to Wako Pure Chemical Industries Ltd., Maruzen Pharmaceuticals Ltd., Morita Kagaku Kogyo Ltd., the Japan Stevia Industry Association, and the Japan Food Additives Association for providing noncommercial steviol glycosides, stevia extract samples, and information on stevia extracts. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Welfare, and Labor of Japan.

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Report

Assessment of Three Methods for the Identification of Enzymatically Hydrolyzed Guar Gum

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Takumi AKIYAMA*, Wakana SEKIGUCHI, Takeshi YAMAZAKI and Hiroshi AKIYAMA

National Institute of Health Sciences:
1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan;
* Corresponding author

Enzymatically hydrolyzed guar gum (EHGG), which is used as a thickener or a soluble dietary fiber, is produced by partial hydrolysis of the guar gum (GG) backbone using mannan endo-β-1,4-mannosidase. In this study, we compared and evaluated 3 methods to distinguish EHGG from other polysaccharides used as food additives or monosaccharides. The first method is based on cross-linking reaction of saccharide hydroxyl groups mediated by borate ions. EHGG showed gelation and was distinguished from some soluble polysaccharides, which did not form gels, and also from polysaccharides with low solubility in water. The second method is based on co-gelation with xanthan gum. It was applicable to GG, but not to EHGG. The third method is based on the alcohol precipitation of hydrophilic polymers. EHGG, some soluble polysaccharides and monosaccharides were dissolved in water at the concentration of 10%, while GG and some polysaccharides were not. The 10% solutions thus obtained were mixed with 2-propanol at the ratio of 1 : 1 (v/v). A white precipitate was formed in the EHGG solutions and the tested soluble polysaccharide solutions, while it was not produced in the monosaccharide solutions. This result demonstrated that soluble polysaccharides including EHGG can be distinguished from polysaccharides with low solubility or monosaccharides by the third method.

Key words: enzymatically hydrolyzed guar gum; polysaccharide; sodium borate; alcohol precipitation

Introduction

Guar gum (GG) is a galactomannan extracted from ground endosperm of guar beans. The basic structure of GG[1] (Fig. 1) is composed of a linear backbone chain of β-1,4-linked mannose residues to which galactose residues are α-1,6-linked at every second mannose. The ratio of mannose:galactose in GG is about 2 : 1 and the average molecular weight is approximately 200,000 Da[1].

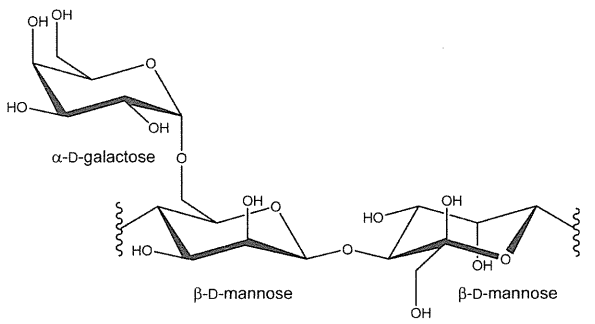

Fig. 1. Partial structure of guar gum (GG) and enzymatically hydrolyzed guar gum (EHGG)

Enzymatically hydrolyzed guar gum (EHGG), which is also called partially hydrolyzed guar gum (PHGG), is produced by partial hydrolysis of the GG backbone with mannan endo-β-1,4-mannosidase. The average molecular weight of EHGG is approximately 20,000 Da[2,3]. EHGG uptake has been shown to suppress postprandial serum lipid levels after consumption of a meal high in fat and cholesterol, reducing the absorption of fat and cholesterol through the depletion flocculation mechanism[4,5]. In addition, EHGG uptake decreased the symptoms in constipation-predominant and diarrhea-predominant forms of irritable bowel syndrome, as well as decreasing abdominal pain[6]. It was reported that diarrhea caused by ingestion of sugar alcohol sweeteners is suppressed by EHGG uptake[7].

EHGG is included in the List of Existing Food Additives in Japan*1 and is classified as a thickener. However, the specification as food additive has not been established by the Ministry of Health, Labour and Welfare of

* akiyamat@nihs.go.jp

*1 Notification No. 120 (Apr. 16, 1996), the Ministry of Health, Labour and Welfare of Japan.
*2 Notice No. 0701007 (Jul. 1, 2005), Department of Food Safety, Pharmaceutical and Food Safety Bureau, the Ministry of Health, Labour and Welfare of Japan.

Japan. Specifications for EHGG as a food additive have not been defined in any country. Therefore, we have been investigating the proposal specification for Japan's Specifications and Standards for Food Additives, including identification tests.

Tests for identification, which can distinguish EHGG from other food additives, are necessary for standardization, and to establish specifications and safety requirements for EHGG. The self-regulatory specification of the Japan Food Additives Association (JFAA) has 2 identification tests for EHGG based on conventional methods for gums[8]. EHGG as a soluble dietary fiber is specified as a standardized food for specialized health uses (standardized FOSHU) in Japan[*2]. Identification tests in this specification are similar to those in the specification of JFAA. To our knowledge, there are no reports on EHGG identification tests in scientific publications.

According to the JECFA specifications for gums, the analysis of individual monosaccharides in gums is recommended for identification tests. We previously developed an identification method for gums by analyzing the diethyldithioacetal derivatives of monosaccharides[9]. However, we considered that this test would be inappropriate as an identification test for routine inspection due to its complexity and time-consuming nature. In the present paper, we compared and evaluated 3 simple methods for use as identification tests for EHGG.

Materials and Methods

Samples and reagents

Gum samples of EHGG, GG and xanthan gum were kindly provided by JFAA. Only 3 EHGG products from 2 manufacturers are circulated by JFAA member companies. Sample A was a product from one manufacturer and samples B and C were from the other.

Other gums were of reagent grade. Pectin from apple, pectin from citrus, swelling alginic acid, non-swelling alginic acid, κ-carrageenan, λ-carrageenan and dextran (molecular weight: 32,000–40,000) were purchased from Wako Pure Chemical Industries, Ltd. Gum arabic from *Acacia senegal* and gum tragacanth were products of Sigma. Gum ghatti and pullulan were purchased from MP Biochemicals and Hayashibara, respectively.

D-Galactose, D-mannose, sodium borate and 2-propanol were of reagent grade, purchased from Wako Pure Chemical Industries, Ltd. Ultrapure water (>18 MΩcm), prepared with a Milli-Q SP Reagent Water System (Millipore, Billerica, U.S.A.), was used throughout the study.

The method based on cross-linking reaction with borate ions

Two grams of gum sample was moistened with 0.4 mL of 2-propanol. Ten milliliters of water was added gently, and the mixture was mixed vigorously until the gum was completely dispersed. Ten milliliters of 5% sodium borate solution was added while mixing gently.

The method based on co-gelation with xanthan gum

Approximately 1, 2 or 4 g of sample was mixed with approximately 1, 2 or 4 g of xanthan gum, and 4 mL of 2-propanol was then added. Two hundred milliliters of water was gradually added with vigorous mixing until the gum was completely dispersed. Approximately half of this solution was transferred to another vessel, heated at 95°C for 10 minutes and then cooled in an ice-water bath.

The method based on precipitation using 2-propanol

Two hundred milligrams of gum sample was transferred to a glass tube and 200 μL of 2-propanol was added. Two milliliters of water was added and mixed well. The 10% mixed monosaccharide solution was prepared by dissolving 74 mg of D-galactose and 148 mg of D-mannose in 2 mL of water. Two milliliters of 2-propanol was added and vigorously mixed.

Results and Discussion

EHGG has high solubility in water because its molecular weight is about one-tenth of that of GG. This property may be utilized to distinguish EHGG from insoluble polysaccharides. On the other hand, its structure as a galactomannan and high molecular weight are useful to distinguish EHGG from some polysaccharides and monosaccharides. In the present paper, we compared and evaluated 3 methods, which are based on different principles, for distinguishing EHGG from polysaccharides used as food additives and monosaccharides.

The applicability of a method based on cross-linking reaction with borate ions (the cross-linking reaction method)

A method based on cross-linking reaction of saccharides via hydroxy group mediated by borate ions was tested. Polyvinyl alcohol, galactomannans and glucomannans are known to crosslink with borate ions. A method based on this property is utilized for the identification test of galactomannans GG and locust bean gum in Japan's Specifications and Standards for Food Additives[10]. In this test, 2 g of a sample is moistened with 4 mL of 2-propanol and then dissolved in 200 mL of water. A 10-mL aliquot of each sample solution, which contains 0.1 g of a sample, is mixed with 2 mL of 5% sodium borate solution. A positive result in this test is judged by the formation of a gel or a significant increase in viscosity. A method based on cross-linking reaction with borate ions is adopted by the identification tests for EHGG in the self-regulatory specifications of JFAA[8] and in the specification as a standardized FOSHU. Twenty grams of a sample is used in these tests.

The applicability of the method based on cross-linking reaction was assessed using all three products circulated by JFAA member companies. In the assessed method, the concentration of a sample and sodium borate were twelve-fold and three-fold higher, respectively, than those in the test for GG because EHGG didn't form a gel in the test for GG. The heating step in the test for GG, which is used to distinguish GG from locust bean gum,

Table 1. Applicability of the cross linking reaction method and the alcohol precipitation method

	Cross linking method		Alcohol precipitation method	
	Solubility in water	Gelation	Solubitily in water	Precipitation
Galactose+mannose	NT	NT	Dissolved	No
EHGG	Dissolved	Yes	Dissolved	Yes
Gum arabic	Dissolved	No	Dissolved	Yes
Dextran	Dissolved	No	Dissolved	Yes
Pullulan	Dissolved	No	Dissolved	Yes
GG	Lump	NT	Paste	NT
Pectin	Paste	NT	Dense suspension	NT
Gum ghatti	Lump	NT	Dense suspension	NT
Xanthan gum	Lump	NT	Paste	NT
Gum tragacanth	Lump	NT	Paste	NT
Swelling alginic acid	Lump	NT	Paste	NT
Non-swelling alginic acid	Paste	NT	Precipitation	NT
κ-Carrageenan	Lump	NT	Precipitation	NT
λ-Carrageenan	Lump	NT	Precipitation	NT

NT: Not tested.

was omitted. Two grams of a sample was moistened with 0.4 mL of 2-propanol and then 10 mL of water was added. Vigorous mixing of the solution completely dissolved samples A and C. Sample B gave a suspension with low viscosity, indicating that the majority of the powder was dissolved. When 10 mL of 5% sodium borate solution was added to the solution, we confirmed that a gel was immediately formed with all samples. Increased viscosity indicated that EHGG has sufficient molecular weight and suitable structure to aggregate when combined with borate.

The applicability of this method was assessed with various polysaccharides used as food additives. To pass the test, a polysaccharide must be dissolved or suspended in water and form a gel when borate solution is added. Out of 36 thickening polysaccharides included in the List of Existing Food Additives in Japan, GG, pectin, gum arabic, gum ghatti, gum tragacanth, alginic acid, κ-carrageenan, λ-carrageenan, dextran and pullulan were obtained. First, 2 g of gum was moistened with 0.4 mL of 2-propanol and then 10 mL of water was added. After vigorous mixing, gum arabic, dextran and pullulan were dissolved. Other gums form lumps or a paste (Table 1). Next, 10 mL of 5% sodium borate solution was added to the solution of gum arabic, dextran and pullulan. Gel was not formed for any sample. There is no report indicating that arabinogalactans or glucans crosslink with borate. These results demonstrate that EHGG is distinguished from polysaccharides with low solubility in water and also from some soluble polysaccharides in this test.

The applicability of a method based on co-gelation with xanthan gum (the co-gelation method)

We next evaluated the applicability of a method based on co-gelation with xanthan gum. A galatomannan generally forms a gel when it is mixed with polysaccharides composed of glucuronic acid, *e.g.*, xanthan gum. This gelation is called co-gelation or viscosity synergy. An identification test in the self-regulatory specifications of JFAA adopts this principle. We examined whether EHGG would induce co-gelation when mixed with xanthan gum. An aliquot of EHGG (sample C) was mixed with xanthan gum. Tested combinations of EHGG and xanthan gum were as follows: (weight of EHGG: weight of xanthan gum)=(1 g : 1 g), (2 g : 1 g), (2 g : 2 g), (4 g : 1 g), (4 g : 4 g). Next, 4 mL of 2-propanol was added, and the mixture was dispersed in 200 mL of water. Approximately half of each mixture was heated at 95°C and then cooled in an ice-water bath. Significant increases in viscosity or gelation were not observed in any of the tested combinations. In contrast, under identical test conditions, the mixing of 1 g GG sample and 1 g xanthan gum resulted in gel formation. Considering this result, it is speculated that the molecular length of EHGG was too small to form an insoluble complex with xanthan gum. EHGG is not distinguished from monosaccharides by this method.

The applicability of a method based on precipitation using 2-propanol (the alcohol precipitation method)

A method based on the alcohol precipitation of hydrophilic polymers was also evaluated. This property of hydrophilic polymers had been utilized in some purification procedures, *i.e.*, the precipitation of plasmid DNA in 50% 2-propanol or in 70% to 80% ethanol. We examined the applicability of a method based on this property for EHGG identification. Two hundred milligrams of GG or EHGG (sample A) was placed in a glass tube, moistened with 200 μL of 2-propanol, and then dissolved in 2 mL of water to make 10% solution. EHGG was completely dissolved in water, while GG resulted in a turbid solution. After 2 mL of 2-propanol was added, a white precipitate was observed. A mixed monosaccharide solution that contains the same ratio and concentration as 10% EHGG solution was tested. A solution containing 3.7% galactose and 7.4% mannose did not produce a white precipitate or any insoluble material upon addition of

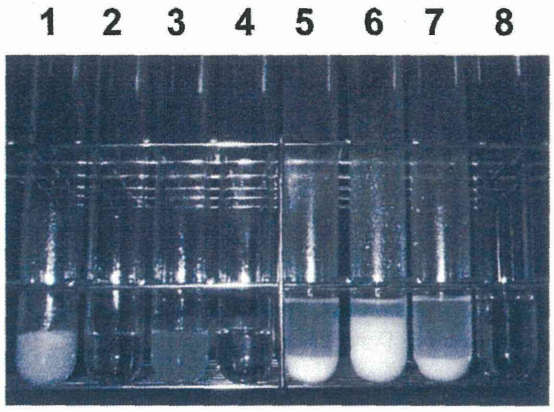

Fig. 2. Precipitation of enzymatically hydrolyzed guar gum (EHGG) in the alcohol precipitation method

1 to 4: without 2-propanol; 1, 10% guar gum; 2 to 4, 10% EHGG (samples A, B and C from the left). 5 to 8: after 2-propanol addition; 5 to 7, 10% EHGG (samples A, B and C from the left); 8, mixed monosaccharide solution containing 3.7% galactose and 7.4% mannose

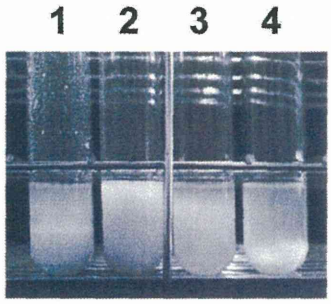

Fig. 3. Precipitation of gums in the alcohol precipitation method

1, 10% EHGG (sample C); 2, 10% gum arabic from *Acacia senegal*; 3, 10% dextran; 4, 10% pullulan

2-propanol. In addition, with this method, the other two products tested (sample B and sample C) produced white precipitates (Fig. 2). Although sample B did not dissolve completely in water (tube 3 in Fig. 2), precipitation (tube 6 in Fig. 2) was clearly distinguishable. These results demonstrate that EHGG, which is a polymer of 100 to 150 sugars, can be distinguished from GG based on solubility in water and also from monosaccharides based on precipitation in 50% 2-propanol using this proposed method.

Other gums, pectin, gum arabic, gum ghatti, gum tragacanth, alginic acid, κ-carrageenan, λ-carrageenan, dextran and pullulan, were tested using this method. Gum arabic, dextran and pullulan were dissolved completely. When 2 mL of 2-propanol was added, a white precipitate was observed for each of these samples (Fig. 3 and Table 1).

It was demonstrated that the alcohol precipitation method was able to distinguish EHGG from GG, polysaccharides with low solubility and monosaccharides.

Conclusion

1) EHGG is distinguished from polysaccharides with low solubility in water and also from some soluble polysaccharides by the cross-linking reaction method.
2) EHGG is not distinguished from monosaccharides by the co-gelation method.
3) The alcohol precipitation method was able to distinguish soluble polysaccharides including EHGG from polysaccharides with low solubility and monosaccharides.

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Note

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Revised method for analyzing 2-acetyl-4-tetrahydroxybutylimidazole in caramel III

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Takumi Akiyama [a], Wakana Sekiguchi [a], Naoki Sugimoto [a], Atsuko Tada [a], Yusai Ito [a], Takeshi Yamazaki [b], Hiroshi Akiyama [a]

a) National Institute of Health Sciences
b) Jissen Women's University

Abstract

Caramel III, a food-coloring additive, is tested in Japan for the presence of the impurity, 2-acetyl-4-tetrahydroxybutylimidazole (THI), using an official HPLC method. In this HPLC method, THI is derivatized with 2,4-dinitrophenylhydrazine and then separated using octyl column. Improvement of the analytical conditions was attempted because contaminants can often compromise this test. Isolation of the analyte was improved when 0.1 mol/L phosphoric acid/methanol mixed solution (70:30) was used as the mobile phase. The revised method gave higher analyte concentrations compared to the standard method. The quantitative values obtained by LC/MS were equivalent to those obtained using the revised method, demonstrating the superiority of the revised method to the standard method.

Keywords : caramel, 2-acetyl-4-tetrahydroxybutylimidazole, 2,4-dinitrophenylhydrazine, HPLC, octyl column

I Introduction

Caramel III, a food-coloring additive, is tested in Japan for the presence of the impurity, 2-acetyl-4-tetrahydroxybutylimidazole (THI), using an official HPLC method. This method requires the derivatization of THI with 2,4-dinitrophenylhydrazine (DNPH, Fig. 1)[1-3]. THI is reported to have immunotoxicity, such as a lymphopenic effect in rats.[4, 5] The standard method is based on the method established by Kröplien et al.[6] Similar methods are defined under standards of JECFA, EU, and FCC[7, 8].

The method comprises purifying THI from caramel III on a column containing two kinds of cation exchange resins, reacting THI with DNPH to derivatize it to hydrazone (THI-DNPH, Fig. 1), and then using an octyl column to isolate and quantify THI-DNPH by HPLC using 0.1 mol/L phosphoric acid/methanol (50:50, v/v) as the mobile phase. However, using the official method, the separation of THI from contaminants is often poor, and the reliability of the quantitative values has been proven problematic. Also, although the JECFA standard recommends an HPLC octyl column with 10 μm particle size,

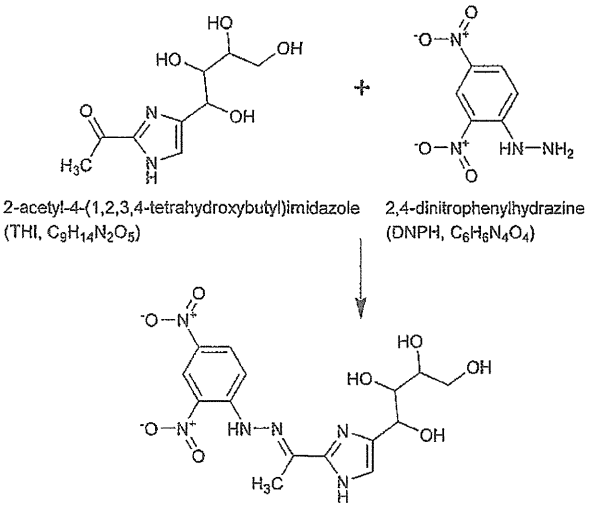

Fig. 1. Structures of 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl) imidazole (THI) and its derivative, 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole 2,4-dinitrophenylhydrazone (THI-DNPH).

Corresponding author: Takumi Akiyama, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan