Table 1
Recoveries of bromate from fresh foods including cabbage, poultry and horse mackerel.

	Added (ng/g)	Found (ng/g)	Recovery (%)
Cabbage	0.0	N.D.	–
	2.0	1.81 ± 0.02	90.7 ± 1.2
	10.0	8.77 ± 0.29	87.7 ± 2.9
Poultry	0.0	Trace[a]	–
	2.0	1.66 ± 0.07	82.9 ± 3.4
	10.0	7.53 ± 0.50	75.3 ± 5.0
Horse mackerel	0.0	N.D.	–
	2.0	1.77 ± 0.15	88.4 ± 7.6
	10.0	7.89 ± 0.08	78.9 ± 0.8

Each value represents the mean ± SD of three individual analyses.
[a] Bromate peak was detected, but the content was less than the lowest quantification limit (0.6 ng/g as bromic acid).

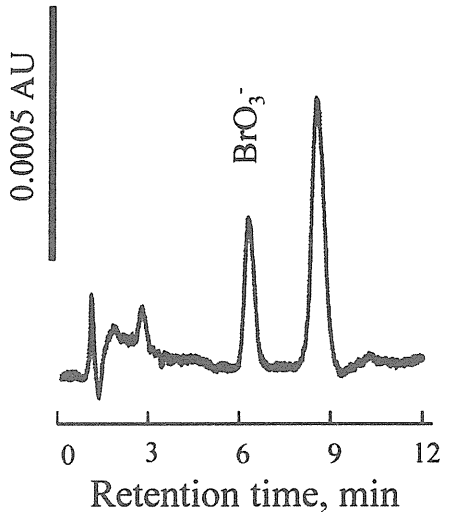

Fig. 4. Chromatogram of drinking water collected at Gunma prefecture in Japan. Fifty millilitres of drinking water were passed through 120 μL of Muromac AG 1X8 ion exchange resin, and the bound bromate on the resin was then eluted by 0.3 M NaCl. Two hundred microlitres of the bromate fraction (volume, 1.0 mL) were subjected to HPLC. The chromatographic conditions are described in Section 2.

with a hypochlorite solution. Sodium hypochlorite solutions have been historically employed as disinfectants for drinking water. In fact, a trace amount of bromate was detected in drinking water, as shown in Fig. 4. Therefore, if bromate penetrates into fresh foods when they are washed with drinking water, the residual bromate can be detected.

4. Conclusion

We established a sensitive, selective and safe HPLC method for the quantification of bromate in fresh foods. The lowest quantitative limit was 0.6 ng/mL (S/N = 5), and the linearity of the correlation between the peak response and bromate concentration was confirmed, even for the lowest quantitative limit (R^2 = 1.000). The sample solutions were prepared from fresh foods through minimal steps as follows: (1) homogenization, (2) centrifugation, (3) ultrafiltration and (4) solid-phase extraction. In the present study, recovery tests for cabbage, poultry and horse mackerel were performed on different days, and the recovery of bromate from these fresh foods at 2 ng/g wet weight ranged from 82.9% to 90.7%; there was no statistical significance. It should be noted that bromate appears to be relatively stable in fresh foods. Through these pretreatment steps, the bromate in 1 g of fresh food was transferred into 1 mL of a 0.3-M NaCl solution. Therefore, the lowest quantitative limit of bromate in fresh foods was 0.6 ng/g of wet weight.

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報 文

食品添加物ステアロイル乳酸ナトリウムのLC-MSによる組成分析

(平成23年7月1日受理)

久保田浩樹[1,*,#] 箕川 剛[1,#] 小関良宏[2] 佐藤恭子[1] 穐山 浩[1]

LC-MS Analysis of Commercial Sodium Stearoyl Lactylate Components

Hiroki KUBOTA[1,#,*], Tsuyoshi MIKAWA[1,#], Yoshihiro OZEKI[2], Kyoko SATO[1] and Hiroshi AKIYAMA[1]

[1] National Institute of Health Sciences: 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan;
[2] Tokyo University of Agriculture and Technology: 2–24–16 Naka-cho, Koganei, Tokyo 184–8588, Japan;
* Corresponding author
# These authors contributed equally to this study.

The components of commercial sodium stearoyl lactylate (SSL), purchased in Japan, were determined and identified using thin layer chromatography (TLC) and liquid chromatography with mass spectroscopy (LC-MS). Stearoyl lactate (SL) and stearoyl-2-lactylate (SLL) were purified using TLC and silica gel chromatography to obtain standards. The results show that SSL consisted of lactic acid (8.4%), stearic acid (15%), SL (57%), and SLL (13%). The total amounts of free lactic acid, lactic acid derived from SL and lactic acid derived from SLL were determined using LC-MS. The mean value was approximately equal to that determined using the JECFA method. This is the first study to determine and identify the components of SSL purchased in Japan, using TLC and LC-MS.

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Key words: ステアロイル乳酸ナトリウム sodium stearoyl lactylate; 薄層クロマトグラフィー TLC; 高速液体クロマトグラフィー質量分析法 LC-MS

緒 言

食品添加物ステアロイル乳酸ナトリウム（以下SSL）(Fig. 1)は，乳化剤，安定剤としてベーカリー製品や菓子類，飲料などの製造加工に広く使用されている．SSLは，欧米諸国では食品添加物ステアロイル乳酸カルシウム（以下CSL）と同じく，古くから食品添加物として使用されてきた．日本においては，CSLは昭和39年に食品添加物に指定されたが，SSLは長年未指定のままであった．

しかしながら，食品安全委員会においてCSLとSSLの安全性は同様であるという評価*1が，SSLの海外での長い使用実績に伴う国際的な汎用性を踏まえて判断され，2010年に食品添加物に指定された．これに伴い，日本で流通する食品添加物SSLを使用した加工食品において，SSLの使用基準を監視する分析法が必要となった．

Joint FAO/WHO Expert Committee on Food Additives (JECFA)はSSLを「ステアロイル乳酸類のナトリウム塩とその関連酸類およびそれらのナトリウム塩との混合物」と定義し，主成分はステアロイルラクトイル乳酸（以下SLL）とパルミトイル乳酸（以下PL）としているが，

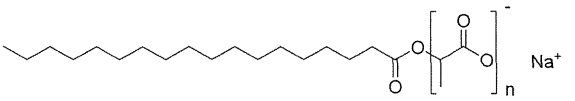

Fig. 1. The structure of sodium stearoyl lactylate (SSL)

The mean value of "n" is normally 2.
Sodium stearoyl-2-lactylate ($n=2$) is a major component of the SSL.

その具体的な成分とその量は示していない[1]．Regulaは薄層クロマトグラフィー(TLC)の解析からSSLが混合物であることを報告[2]し，さらにSudraudらがSSLの成分が，パルミチン酸，PL，ミリスチン酸，ミリストイル乳酸，ステアリン酸，ステアロイル乳酸（以下SL），SLLであることを報告[3]している．しかしながら，いまだSSLの量的な成分比を明らかにした報告はない．

SSLは，生体内で乳酸に代謝されることが明らかになっているが，乳酸に関しては生殖発生毒性が報告*1されていることから，SSLから生じる乳酸の量に関しては詳細に解析することが安全性データにおいても重要であると考えられる．

* 連絡先 kubota@nihs.go.jp
[1] 国立医薬品食品衛生研究所：〒158–8501 東京都世田谷区上用賀1–18–1
[2] 東京農工大学：〒184–8588 東京都小金井市中町2–24–16

*1 添加物評価書 ステアロイル乳酸ナトリウム 厚生労働省発食安 第0206001号 平成20年7月10日

そこで本研究では，わが国で流通しているSSLの成分を分離精製し，それら成分をLC-MSを用いて詳細に質的，量的に解析したので報告する．

実験方法

1. 試　　薬

SSLは食品添加物協会通じて入手した武蔵野化学(株)製の2ロット (Lot. No. SLN71014, SLN71015) を用いた．本品は帯黄白色をした破砕状の粉末であった．乳酸リチウム（純度99%以上）はACROS社製，ステアリン酸（純度98%以上）は東京化成工業㈱製を用いた．シリカゲル薄層プレートは，分析用にはMerck社製TLC（Silica gel 60 F_{254}，20 cm×20 cm，膜厚0.25 mm），分取用には同社製PTLC（Silica gel 60 F_{254}, 20 cm×20 cm，膜厚2 mm）を用いた．シリカゲルクロマトグラフィーのカラム充填剤にはMerck社製Silica gel 60（粒径0.040～0.063 mm）を用いた．ディスポーサブルメンブランフィルターは日本ミリポア社製(Millex®-LG, 0.2 μm)，LC-MSの移動相にはHPLC用を使用した．その他試薬および溶媒は市販品の特級試薬を使用した．

2. 装　　置

高速液体クロマトグラフ質量分析計(LC-MS): Waters AQUITY UPLC Quattro Premier：ウォーターズ社製．

LC-MS条件

(1) LCカラム：Inertsil C8 (5 μm, 2.1 mm×150 mm) (ジーエルサイエンス(株)製)，移動相：0.1%ギ酸溶液–アセトニトリル(10 : 90)，カラム温度：40℃，流速：0.2 mL/min，注入量：5 μL

(2) MS

イオン化法：ESI (negative)，キャピラリー電圧：3.00 kV，コーン電圧：30 V，エクストラクター電圧：3.00 V，ソース温度：120°C，脱溶媒温度：350°C，コーンガス流量：50 L/hr，脱溶媒ガス流量：350 L/hr，質量走査範囲：m/z 80～500.

乳酸，ステアリン酸，SL，SLLの擬分子イオン $[M–H]^-$ を以下のようにシングルイオンモニタリング法(SIM)ターゲットイオンとした．

乳酸：m/z 89 $[CH_3–CH(OH)COO]^-$

ステアリン酸：m/z 284 $[CH_3(CH_2)_{16}–COO]^-$

SL: m/z 355 $[CH_3(CH_2)_{16}–COO–CH(CH_3)–COO]^-$

SLL: m/z 427 $[CH_3(CH_2)_{16}–COO–(CH(CH_3)–COO)_2]^-$

3. SSL成分の分離および同定

Regulaの報告[2]を参考に，薄層クロマトグラフィー(TLC)でSSL成分の分離を検討した．SSLのクロロホルム溶液(0.5 g/mL) 1 μLをシリカゲルTLCプレートに供し，石油エーテル–ジエチルエーテル–酢酸(100 : 100 : 1)混液で展開した．ブロモクレゾールグリーン(BCG)試薬を噴霧して，カルボキシル基を持つ化合物を検出（黄色に発色）し，さらにヨウ素チャンバーにTLCプレートを静置して炭化水素化合物を検出（褐色に発色）し，それぞれのスポットのRf値を求めた．

SSL成分のうち，乳酸とステアリン酸は市販の標準品があるため，それらの標準溶液をSSLと同時にTLCに供してスポットの同定を行った．ステアリン酸標準品としてステアリン酸のエタノール溶液(500 mg/mL)および乳酸標準品として乳酸リチウムの水溶液（乳酸として500 mg/mL）を調製した．

市販の標準品がないSL, SLLに関しては，分取用PTLCプレートから呈色したスポットのRf値に相当する未発色の領域をかき取り，分取精製し，LC-MSのスキャン測定に供してその領域に含まれる化合物の同定をした．分取方法は以下のとおりである．分取用PTLCプレートの下端から1 cm，両端から1 cmの領域にSSL (Lot. No. SLN71015)のクロロホルム溶液(0.5 g/mL) 500 μLをTLCプレートの下端から1 cmの位置に線状に付した．石油エーテル–ジエチルエーテル–酢酸(100 : 100 : 1)混液で展開した．展開後，プレート両端から2 cmより内側のシリカゲル表面をアルミホイルで覆い，両端より2 cmの領域にBCG試薬を噴霧した．両端の発色したスポット間にあるアルミホイルで覆ってあった未発色のままの領域をかき取った．かき取ったシリカゲルをクロロホルム約20 mLで抽出し，濃縮乾固した．得られた残渣をエタノール1 mLに溶解し，メンブランフィルターでろ過したものをLC-MSに供する試験液とした．

4. SLおよびSLLの精製

SSL中のSL，SLL含有量を検討するために，SLおよびSLLをSSLから精製し，定量用の標準品とした．

SSL 0.2 gをクロロホルム1 mLに溶解させ，その全量をシリカゲルカラムに供した．溶出液の最初10 mLを採った後，溶出液を1 mLずつ試験管に分取した．カラムサイズおよび溶出条件は以下のとおりである．

カラムサイズ：1.5 cm×30 cm，充填剤：シリカゲル，移動相：石油エーテル–ジエチルエーテル–酢酸(100 : 100 : 1)，流速：自然落下．シリカゲル10 gを移動相と同液で湿式充填した．

5. LC-MSによるSSL成分の定量

SSL 50 mgを精密に量り採り，エタノール50 mLに溶解して1.0 mg/mLに調製した．さらにエタノールで100 μg/mLに正確に希釈してSSL試験液とした．SSL成分を定量する際の標準品には，市販のステアリン酸，乳酸リチウムおよび，精製して得たSL, SLLを用いた．乳酸リチウムは水溶液として1.0 mg/mLに調製し，SL, SLL, ステアリン酸はエタノール溶液として1.0 mg/mLに調製してそれぞれを標準原液とした．標準原液をそれぞれ1～100 μg/mLに希釈した検量線用標準液をLC-MSに供し，SIM測定で得られるピーク面積から検量線を作成した．SSL試験液を同LC-MS条件に供し，含まれる各成分を定量した．

6. SSLの総乳酸試験

JECFAのステアロイル乳酸ナトリウム成分規格の総乳

酸試験[1]に従い，SSLの総乳酸値を求めた．総乳酸試験とは，SSLをけん化してSLおよびSLLの構成成分である乳酸とステアリン酸に分解し，SSLを構成する乳酸の総量を求める試験である．総乳酸試験で得たSSL中の乳酸量と，LC-MSで定量したSL，SLL由来の乳酸およびSSL中のフリーの乳酸の合算量を比較検討した．

結果および考察

1. SSL成分の分離

SSLをTLCに供した結果，BCG試薬およびヨウ素に発色を示す3つのスポットA，B，C（*Rf*値0.7，0.3，0.1）および原点のスポットDを検出した．ステアリン酸標準品の*Rf*値はスポットAと一致し，乳酸標準品のスポットは原点のスポットDと同様に展開されなかった(Fig. 2)．

さらに分取用TLCからスポットB，Cを分取し，HPLC分析とLC-MSのトータルイオンクロマトグラフィー(TIC)でそれぞれが単一のピークとして検出された．その結果，BとCの化合物に関しては単一化合物であると示唆された．それぞれのマススペクトルを解析したところ，スポットBからは*m/z*: 283，355を検出し，スポットCからは*m/z*: 283，355，427を検出した．SSLの原材料および製法と過去の報告から鑑みて，スポットBはSLで，検出された*m/z*: 355はSLが脱プロトンした擬分子イオン[M−H]$^-$であり，*m/z*: 283はSLが脱乳酸化したフラグメントイオン[$CH_3(CH_2)_{16}$−COO]$^-$であると判断した．またスポットCはSLLで，検出された*m/z*: 427は，SLLが脱プ

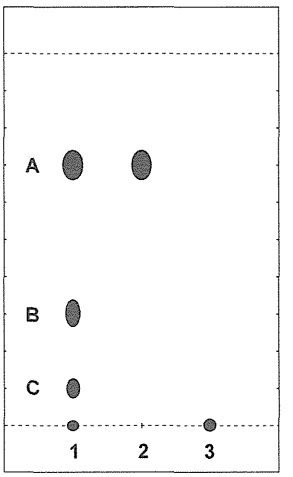

Fig. 2. TLC analyses of SSL extract and reference samples

SSL was extracted with chloroform and separated on a silica gel plate using petroleum ether–diethyl ether–acetic acid (100 : 100 : 1) as the developing solvent. Lane 1, SSL extract; Lane 2, stearic acid ethanol solution (500 mg/mL); Lane 3, lithium lactate solution (500 mg/mL).

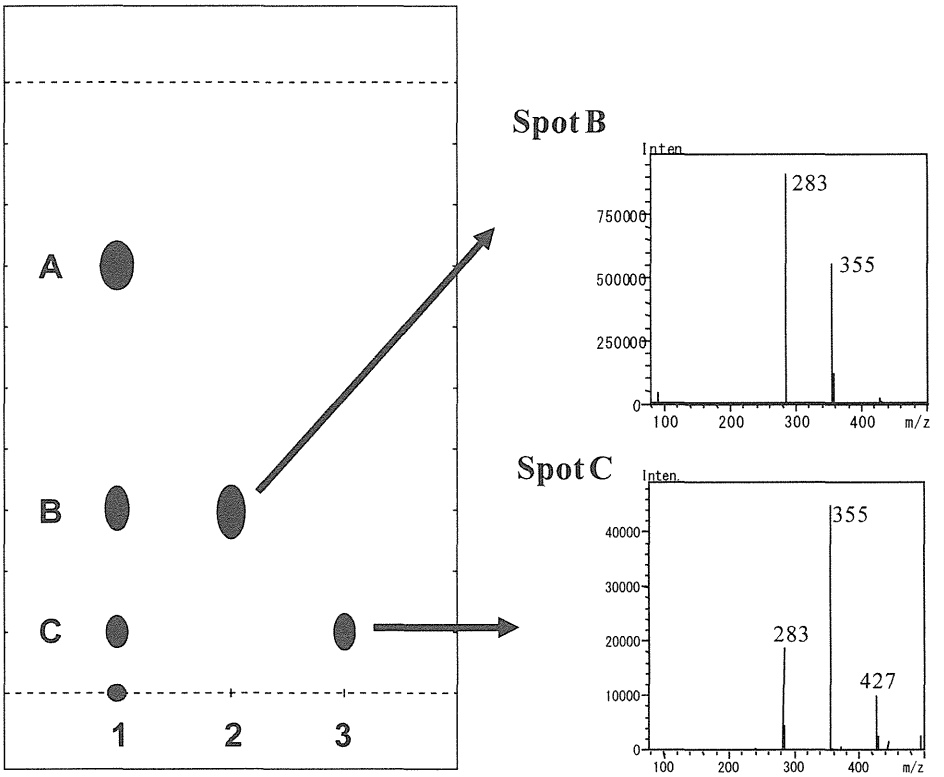

Fig. 3. Preparative TLC and LC-MS analysis of SSL extract

(Left) Preparative TLC chromatogram of SSL extract. The SSL was extracted with chloroform and separated on a silica gel plate using petroleum ether–diethyl ether–acetic acid (100 : 100 : 1) as the developing solvent. Lane 1, SSL extract; Lane 2, Spot B fraction; Lane 3, Spot C fraction

(Right) The LC-MS spectrum of Spots B (upper) and C (bottom). Spots B and C were concluded to be stearoyl lactate (SL) and stearoyl-2-lactylate (SLL), respectively.

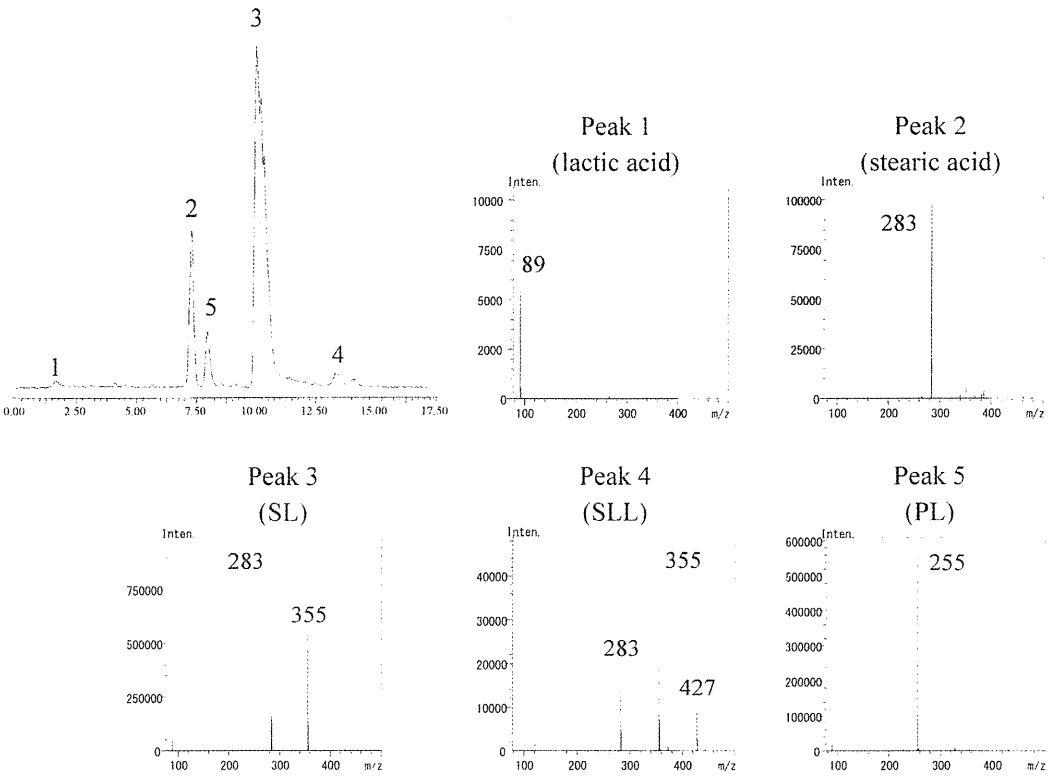

Fig. 4. LC-MS total ion chromatogram of SSL and MS spectra of each peak

ロトンした擬分子イオン$[M-H]^-$であり，*m/z*: 283はSLLが脱ラクチル乳酸化したフラグメントイオン$[CH_3(CH_2)_{16}-COO]^-$であり，*m/z*: 355はSLLが脱乳酸化したフラグメントイオン$[CH_3(CH_2)_{16}-COO-CH_2(CH_3)COO]^-$であると判断した．これらの結果から，国内に流通するSSLは乳酸，ステアリン酸，SL，SLLを含有することが示された(Fig. 3).

2. SSLからのSLおよびSLLの精製

SSL成分をシリカゲルカラムクロマトグラフィーに供し，分離精製を試みた．

シリカゲルカラムクロマトグラフィーの溶出液を1 mLずつ試験管20本に分取した．それら各溶出液2 μLをTLCに展開し，ヨウ素発色を確認した．試験管2～5本目の画分は*Rf*値0.7を示し，ステアリン酸と同一の*Rf*値を示した．これらの画分に含まれる成分はステアリン酸であると推定された．試験管8～11本目の画分は，LC-MS分析のトータルイオンクロマトグラムで単一ピークであり，そのピークのマススペクトルでは，前述の*Rf*値0.3を示すスポットBと同じく*m/z*: 283, 355が検出されたことにより，SLであることが確認された．それらの画分を集めて窒素ガスで濃縮乾固し，95 mgの白色結晶を得た．さらに試験管12～20本目の画分は，LC-MS分析のトータルイオンクロマトグラムで単一ピークであり，そのピークのマススペクトルでは前述の*Rf*値0.1を示すスポットCと同様に*m/z*: 283, 355, 427が検出されたことにより，SLLであることが確認された．それらの画分を集めて濃縮乾固する

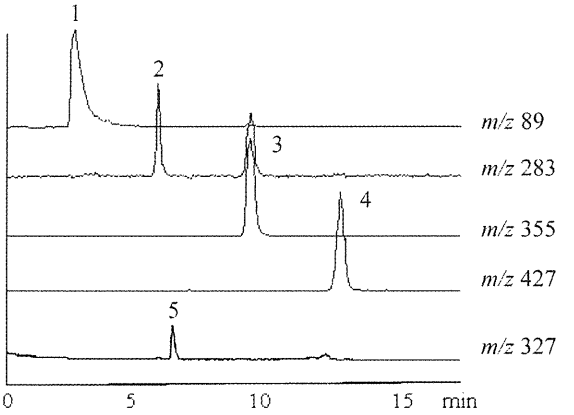

Fig. 5. SIM chromatogram of SSL

1) lactic acid (*m/z* 89), 2) stearic acid (*m/z* 283), 3) SL (*m/z* 355), 4) SLL (*m/z* 427) SSL, 5) PL (*m/z* 327) concentration: 100 μg/mL

Table 1. Amounts of lactic acid, stearic acid and related compounds determined in SSL

Sample Lot.	Lactic acid (%)	Stearic acid (%)	Stearoyl lactylate (%)	Stearoyl-2-lactoyl lactylate (%)
SLN71014	8.3±0.1	14.3±1.7	56.0±1.4	11.2±0.3
SLN71015	8.4±0.5	15.2±0.9	58.3±1.1	14.3±1.6
Mean	8.4	14.8	57.2	12.8

Mean±S.D. ($n=3$)

Table 2. Total amount of lactic acid detected in SSL determined by the colorimetric method (JECFA method) and LC-MS

Sample Lot.	Colorimetric method	LC-MS method			
	Total lactic acid (%)	Free lactic acid (%)	Lactic acid in SL (%)	Lactic acid in SLL (%)	Total lactic acid (%)
SLN71014	30.0	8.3	14.6	4.9	27.8
SLN71015	29.6	8.4	14.8	5.7	28.9
Mean	29.8	8.4	14.7	5.3	28.4

と15 mgの白色結晶を得た．両白色結晶のトータルイオンクロマトグラムには夾雑ピークが観察されなかったことから高純度のSLおよびSLL精製物を得たと考えられた．

3. LC-MSによるSSL成分の定量

SLとSLLの高純度精製物を得たことにより，SSL試験液をLC-MSで分析し，各成分を定量することを試みた．SSLのトータルイオンクロマトグラムを示す(Fig. 4)．乳酸標準品，ステアリン酸標準品，SL精製物，SLL精製物の保持時間と各ピークのマススペクトルから，ピーク1は乳酸，ピーク2はステアリン酸であることが明らかとなった．ピーク3，4はSL精製物，SLL精製物からの同様の解析からピーク3はSL，ピーク4はSLLと判断した，ピーク5は微量の単一ピークで，そのマススペクトルを解析したところ，m/z 255が検出され，これはパルミチン酸の分子イオン$[CH_3(CH_2)_{14}COO]^-$と一致した．さらに，SIMを用いてPLの擬似分子イオン$[M-H]^-$に相当するm/z 327で測定したとき，同保持時間にピークが検出されたことからPLと推定された(Fig. 5)．

さらに乳酸標準品，ステアリン酸標準品およびSL精製物，SLL精製物で作成した検量線からSSLの成分を定量した(Table 1)．SSL 100 μg/mL中に含まれるフリーの乳酸は8.4%，ステアリン酸は14.8%，SLは57.2%，SLLは12.8%であった．これら4成分はSSL重量の93.1%を占めた．SSL中に含有が認められたPLについては，市販のPL標準品はなく，SSLからの精製では標準品としての量的な確保が困難であったために，その定量に至らなかった．

4. LC-MSによる定量値から算出するSSL中の乳酸量と総乳酸値との比較

JECFA法で測定したSSLの総乳酸量とLC-MSの定量値から算出したSL，SLL由来する乳酸量を比較した(Table 2)．

SL由来の乳酸量は，SL分子中の乳酸分子比（乳酸分子量/SL分子量＝0.25）を測定されたSL量に乗じて求めた．SLL由来の乳酸量も同様に，測定されたSSL中のSLL量にSLL分子中の乳酸分子比（乳酸分子量×2/SLL分子量＝0.42）を測定されたSLL量に乗じて求めた．

SSL（100 μg/mL）2ロットの各測定値の平均値の解析においてSL，SLLに由来する乳酸量(14.7 μg/mL，5.3 μg/mL)とフリーの乳酸量(8.4 μg/mL)の合算値は28.4 μgであり，SSL重量の28.4%を占めた．その値はSSLの総乳酸試験で得た総乳酸値(29.8%)に近似する値を示した．

LC-MSで解析したSSL中のフリーの乳酸，SL，SLL量から算出したSSL中の乳酸量は，JECFAの総乳酸試験で得られたSSLの総乳酸値と合致したことから，本研究により初めてSSL成分を量的，質的に評価した．

ま と め

JECFAのSSL規格ではSLLとPLが主成分であるとされているが[1]，その詳細を示す報告はない．日本に流通しているSSLを分析した結果，SLが58%で主成分であることが判明した．その他の成分はステアリン酸，SLL，乳酸，PLであることが初めて明らかとなった．

世界的に汎用性のある食品添加物に関してはJECFAの規格と日本の規格を整合する必要があると考えられる．そのため，海外に流通するSSLにおいても，本研究と同様に成分を解析することが望まれる．

謝　辞

著者の久保田浩樹と箕川　剛の本研究における貢献度は同等である．本研究は，厚生労働科学研究補助金により実施した．食品添加物ステアロイル乳酸ナトリウムをご提供いただいた武蔵野化学株式会社に深謝いたします．

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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].

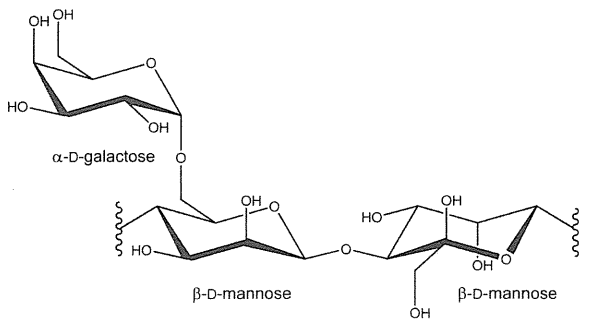

Fig. 1. Partial structure of guar gum (GG) and enzymatically hydrolyzed guar gum (EHGG)

* akiyamat@nihs.go.jp

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

*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

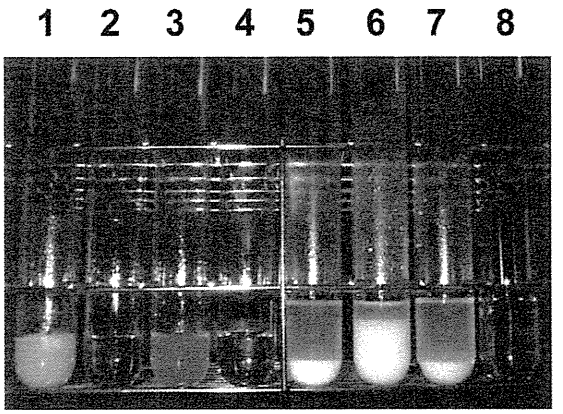

Fig. 2. Precipitation of enzymatically hydrolyzed guar gum (EHGG) in the alcohol precipitation method

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

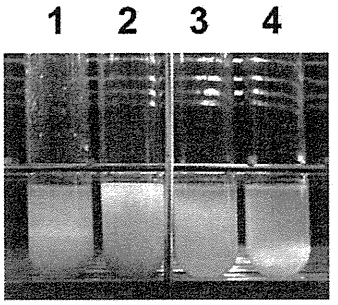

Fig. 3. Precipitation of gums in the alcohol precipitation method

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,

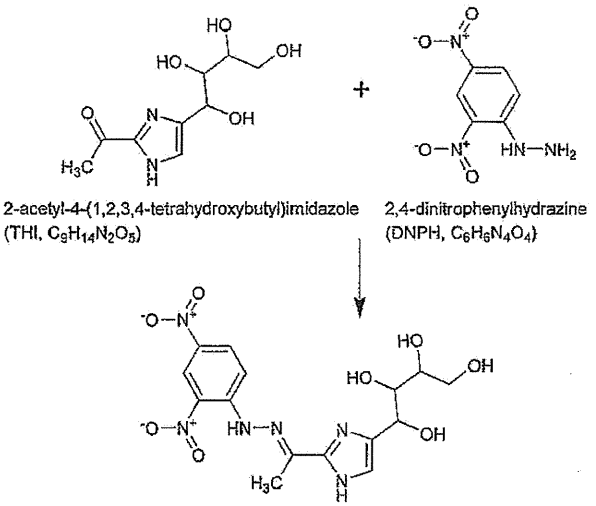

Fig. 1. Structures of 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl) imidazole (THI) and its derivative, 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole 2,4-dinitrophenylhydrazone (THI-DNPH).

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

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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

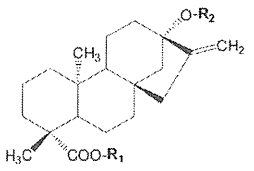
1. Introduction

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, $W_{stv\text{-}stn}$ is the weight of the stevioside standard (dried basis), W_{smp} is the weight of the sample (dried basis), $A_{x\text{-}smp}$ is the peak area of the steviol glycoside (x) in the sample solution, $A_{stv\text{-}stn}$ 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, $W_{reA\text{-}stn}$ is the weight of the rebaudioside A standard (dried basis), W_{smp} is the weight of the sample (dried basis), $A_{reA\text{-}smp}$ is the peak area of rebaudioside A in the sample solution, and $A_{reA\text{-}stn}$ 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.

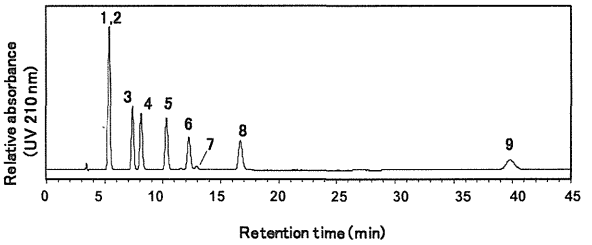


Figure 2. HPLC chromatogram of the reference standard mixture of nine steviol glycosides using an amino column as described in the conventional JECFA method. Separation was carried out on an supelcosil LC-NH2 column (4.6 mm i.d. × 250 mm, 5 μm) at a column temperature of 40°C using an 80:20 (v/v) mixture 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.

3.3. Calibration Curves for the Developed Assay Method

The calibration curves were constructed for a range of

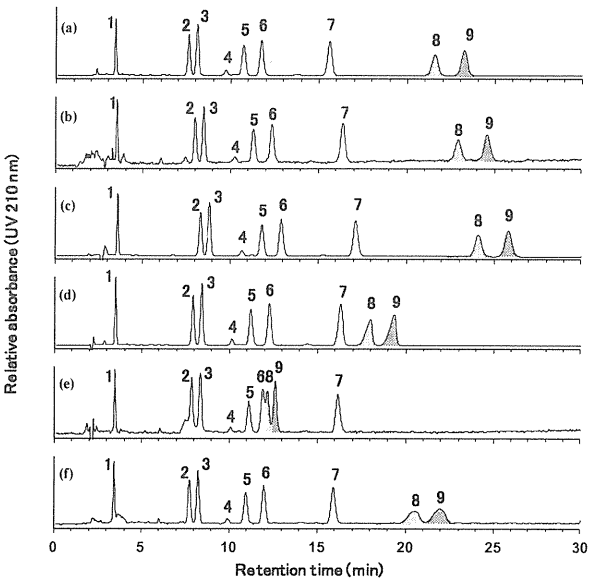


Figure 3. HPLC chromatograms of the reference standard mixtures of nine steviol glycosides obtained using an ODS column with six different mobile phases. the mobile phases were 32:68 (v/v) mixtures of acetonitrile and various solutions, including (a) 10 mmol/L sodium phosphate buffer (pH 2.6), (b) 1.4 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.

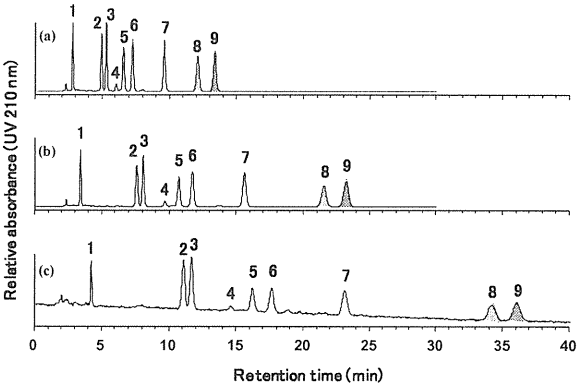


Figure 4. HPLC chromatograms of the reference standard mixture of nine steviol glycosides using different mobile phases. Mobile phases were 35:65 (v/v) mixtures of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) (a), 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (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.

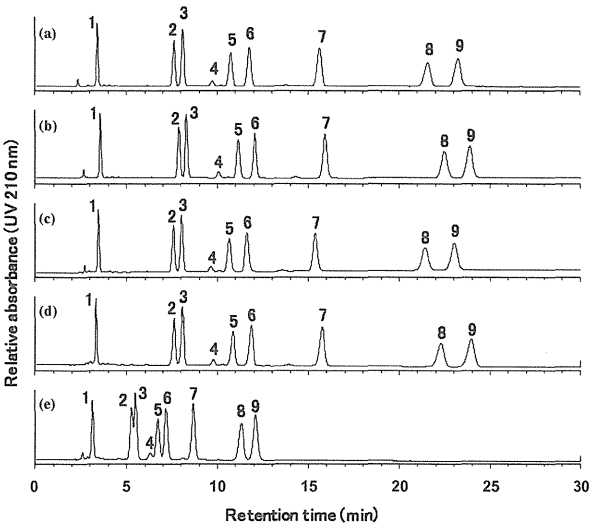


Figure 5. HPLC chromatograms of the reference standard mixture of nine steviol glycosides using different ODS columns (4.6 × 250 mm, 5 μm), Capcell Pak C18 MGII (a), Shim-pack CLC-ODS (b), Luna C18(2) (c), SunFire C18 (d), or other ODS column (e). Mobile phase was 32:68 (v/v) mixture of acetonitrile 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.

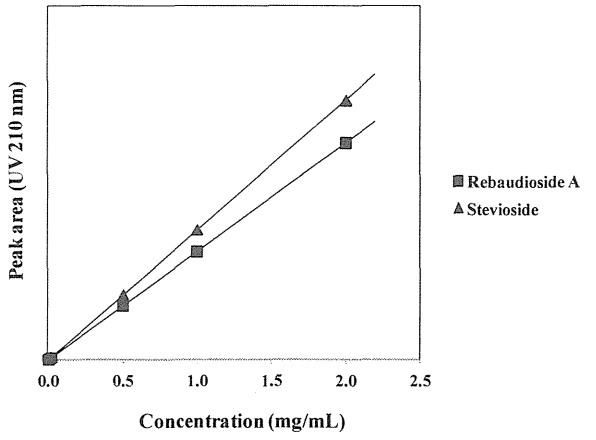


Figure 6. Calibration curves for the stevioside and rebaudioside A standards.

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

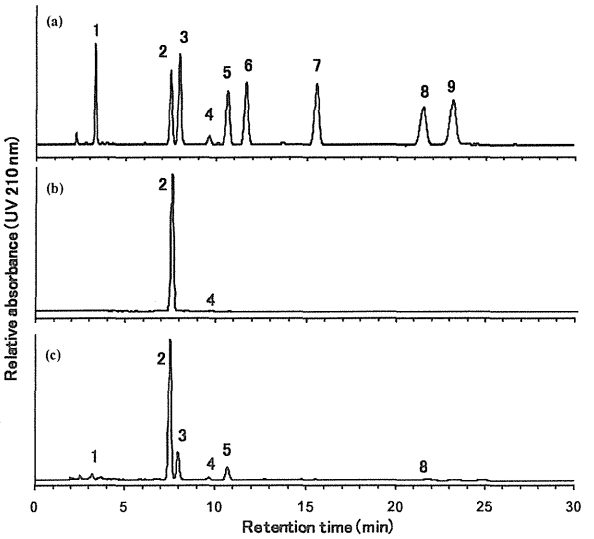


Figure 7. HPLC chromatograms of the reference standard mixture of nine steviol glycosides (a), stevia extracts used as food additives, RA 95 (b) and RA 60 (c). Separation was carried out on a Capcell Pak C18 MGII column (4.6 mm × 250 mm, 5 μm) at a column temperature at 40°C and a flow rate of 1.0 mL/min. 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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Quantitative Determination of Carthamin in Carthamus Red by ^{1}H-NMR Spectroscopy

Takamitsu Yoshida,[a,†] Kazuyoshi Terasaka,[a] Setsuko Kato,[a] Fan Bai,[a] Naoki Sugimoto,[b] Hiroshi Akiyama,[b] Takeshi Yamazaki,[c] and Hajime Mizukami*,[a]

[a] *Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan:* [b] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan: and* [c] *Faculty of Human Life Sciences, Jissen Women's University; 4–1–1 Osakaue, Hino, Tokyo 191–8510, Japan.*
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Carthamus Red is a food colorant prepared from the petals of *Carthamus tinctorius* (Asteraceae) whose major pigment is carthamin. Since an authentic carthamin standard is difficult to obtain commercially for the preparation of calibration curves in HPLC assays, we applied ^{1}H-NMR spectroscopy to the quantitative determination of carthamin in commercial preparations of Carthamus Red. Carthamus Red was repeatedly extracted in methanol and the extract was dissolved in pyridine-d_5 containing hexamethyldisilane (HMD) prior to ^{1}H-NMR spectroscopic analysis. The carthamin contents were calculated from the ratios of singlet signal intensities at approximately σ: 9.3 derived from H-16 of carthamin to those of the HMD signal at σ: 0. The integral ratios exhibited good repeatability among NMR spectroscopic analyses. Both the intra-day and inter-day assay variations had coefficients of variation of <5%. Based on the coefficient of absorption, the carthamin contents of commercial preparations determined by ^{1}H-NMR spectroscopy correlated well with those determined by colorimetry, although the latter were always approximately 1.3-fold higher than the former, irrespective of the Carthamus Red preparations. In conclusion, the quantitative ^{1}H-NMR spectroscopy used in the present study is simple and rapid, requiring no carthamin standard for calibration. After HMD concentration has been corrected using certified reference materials, the carthamin contents determined by ^{1}H-NMR spectroscopy are System of Units (SI)-traceable.

Key words quantitative NMR; carthamin content; Carthamus Red; *Carthamus tinctorius* (Asteraceae)

Carthamus Red is a natural colorant, which is produced from the dried petals of *Carthamus tinctorius* L. (Asteraceae). It has been permitted for use and is distributed in Japan because it is accepted as a food additive on the List of Existing Food Additives prepared by the Ministry of Health, Labour and Welfare of Japan.[1] The major pigment in Carthamus Red is carthamin with two fully conjugated chalcone moieties,[2,3] whereas a minor red pigment was isolated and identified as the hydroxyethyl ether of carthamin[4] (Fig. 1A). Carthamin was reported to exhibit potent radical-scavenging and neuroprotective activities.[5] The carthamin content is an important index for the evaluation and/or validation of commercial preparations of Carthamus Red. An HPLC method was described for the quantitative determination of carthamin.[6] Nevertheless, a colorimetric index based on the color value has been used to validate the Carthamus Red preparations[7] because it is difficult to obtain commercially an authentic carthamin sample to prepare the calibration curves.

NMR spectroscopy is a powerful tool for structure elucidation and quantitative determination of organic molecules.[8] The basis of proton-specific quantitative NMR (qHNMR) spectroscopy is that the integral of the signal (area under the signal) is proportional to the number of protons producing the signal and that the signal intensity per proton is proportional to the molar amount of the compound. qHNMR spectroscopy is unique because it allows absolute quantification of organic compounds by comparing the signal intensity of a specific compound and that of an appropriate internal standard with known absolute purity, and the value obtained is theoretically traceable using the International System of Units (SI-traceable).[9,10] Compared with HPLC, qHNMR spectroscopy is advantageous because no standard compounds are required to prepare calibration curves. Furthermore, qHNMR spectroscopy is rapid and non-invasive, and in most cases it does not require laborious sample pre-cleaning steps.[11]

In the present investigation, we demonstrated that qHNMR spectroscopy can be effectively applied for the quantitative determination of carthamin in commercial preparations of Carthamus Red without an authentic carthamin sample.

Experimental

Isolation of Carthamin Carthamus Red (color value 8000; Yaegaki Bio-Industry, Inc., Himeji, Japan) was stirred with methanol at room temperature for 3 h. The methanol extract was applied to octadecylsilyl silica gel (ODS) column which was then eluted with 60% methanol containing 0.2% formic acid. Red pigment fraction thus obtained was purified by repeating ODS column chromatography to yield carthamin. Purity of carthamin was estimated to be 42.6±1.1% by qHNMR method.

Carthamus Red Preparations Commercial preparations of Carthamus Red were obtained from various suppliers (Table 1). The voucher samples were stored in the Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University. The color values of preparations were determined by a previously described method.[12]

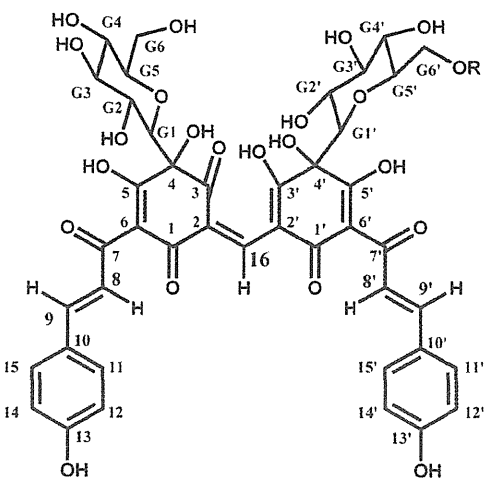
Chemicals Pyridine-d_5 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hexamethyldisilane (HMD, Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) was used as an internal standard for NMR spectroscopic analyses.

The authors declare no conflict of interest.

† Present address: *R&D Center, Matsuura Yakugyo Co., Ltd., 77–2 Torashinden, Oodakacho, Midori-ku, Nagoya 459–8001, Japan.*

* To whom correspondence should be addressed. e-mail: hajimem@phar.nagoya-cu.ac.jp

(A)



R=H: Carthamin
R=CH_2CH_2OH: Carthamin hydroxyethyl ether

(B)

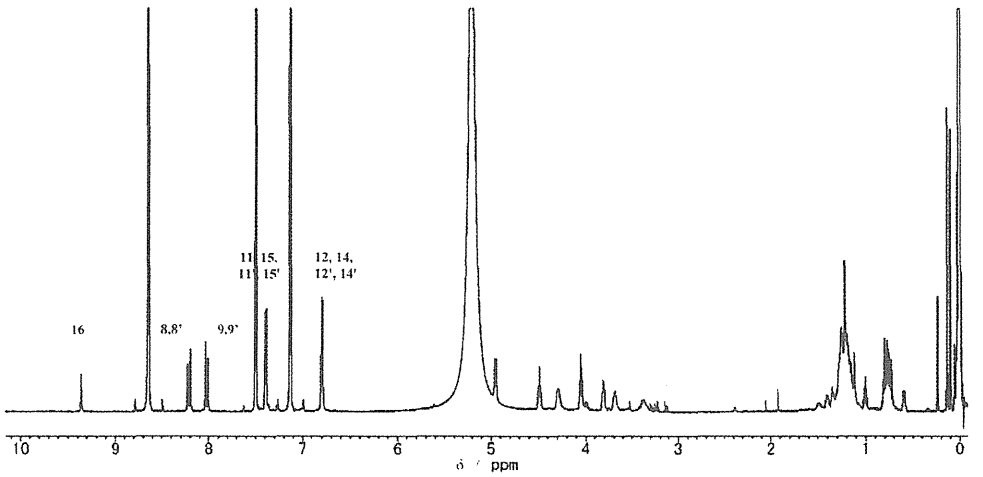


Fig. 1. (A) Chemical Structures of Carthamin and Carthamin Hydroxyethyl Ether and (B) ^{1}H-NMR Spectrum of Carthamin Prepared from Carthamus Red

Carthamin was dissolved in pyridine-d_5 containing hexamethyldisilane (HMD) as a NMR standard.

Table 1. Commercial Preparations of Carthamus Red Used in the Present Investigation

Carthamus Red	Lot No.	Color value	
		Claimed	Estimated[a]
Product No. 1	09A012	NA[b]	18.4±0.8
Product No. 2	90625	13.8	13.9±0.3
Product No. 3	90612	NA	0.143±0.02
Product No. 4	90701	7.43	8.19±0.99
Product No. 5	90528	0.455	0.354±0.02

a) Average±standard deviation from five independent measurements. *b*) Not available.

Potassium hydrogen phosphate (PHP; NMIJ CRM 3001-a), a certified reference material whose purity was certified to be 100.00±0.027%, was purchased from Wako Pure Chemical Industries, Ltd. 1,4-Bis(trimethylsilyl)benzene-d_4, a reference material for qHNMR spectroscopy whose purity was certified to be 99.8% according to the National Metrology Institute of Japan (NMIJ), was obtained from Wako Pure Chemical Industries, Ltd.

^{1}H-NMR Spectroscopy Apparatus and Parameters The ^{1}H-NMR spectra were recorded using a Bruker Avance 600 (600 MHz) spectrometer. NMR spectroscopy acquisition and processing were performed as previously described.[11] In brief, eight scans were performed for each sample with a 90° pulse and a 30-s pulse delay because high precision qHNMR spectra can be achieved when the pulse angle is 90° and the pulse delay time is greater than the quintuple spin-lattice relaxation time (>5*T_1). The chemical shifts are given as δ values (ppm) relative to the internal standard HMD. The start and end points were manually selected during the integration of the signal.

Determination of HMD Concentration in the qHNMR