ノート

既存添加物ウルシロウの成分分析

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Analysis of Constituents in Urushi Wax, a Natural Food Additive

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Urushi wax is a natural gum base used as a food additive. In order to evaluate the quality of urushi wax as a food additive and to obtain information useful for setting official standards, we investigated the constituents and their concentrations in urushi wax, using the same sample as scheduled for toxicity testing. After methanolysis of urushi wax, the composition of fatty acids was analyzed by GC/MS. The results indicated that the main fatty acids were palmitic acid, oleic acid and stearic acid. LC/MS analysis of urushi wax provided molecular-related ions of the main constituents. The main constituents were identified as triglycerides, namely glyceryl tripalmitate (30.7%), glyceryl dipalmitate monooleate (21.2%), glyceryl dioleate monopalmitate (2.1%), glyceryl monooleate monopalmitate monostearate (2.6%), glyceryl dipalmitate monostearate (5.6%), glyceryl distearate monopalmitate (1.4%). Glyceryl dipalmitate monooleate isomers differing in the binding sites of each constituent fatty acid could be separately determined by LC/MS/MS.

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Key words: 食品添加物 food additive; ウルシロウ Urushi wax; トリグリセリド triglyceride; 脂肪酸 fatty acid; ガスクロマトグラフィー/質量分析法 GC/MS; 液体クロマトグラフィー/質量分析法 LC/MS

緒 言

漆ろうは木ろうとともに，日本古来の天然ワックスであり，日常の灯火，神仏事の灯明には欠かせないロウソクの原料として珍重されてきたものであるが，天然食品添加物としてもガムベースまたは光沢剤として，チューインガム，キャンディや果実などに用いられる．天然食品添加物として用いられる漆ろうは，既存添加物名簿[1]に「ウルシロウ」の名称で収載され，「ウルシの果実から得られた，グリセリンパルミタートを主成分とするものをいう．」と定義されている．また，その基原・製法・本質として，「ウルシ科ウルシ (*Rhus verniciflua* LINNE) の果実より，融解，さらして得られたものである．主成分はグリセリンパルミタートである．」と記載されている[2]．また，既存添加物名簿収載品目リスト注解書[3]には，「主に中国産のウルシの実の種子の中果皮より抽出されたトリグリセリドを漂白精製したもので，正式には油脂に分類される物質である．成分はパルミチン酸などのモノカルボン酸のグリセリンエステルが主体であるが，ジカルボン酸のグリセリンエステルも含有する．」と概説されている．しかし，食品添加物としてのウルシロウは，業界自主品質規格[4]が設定されているものの，成分組成や安全性についての報告はなく，食品添加物公定書にも未収載である．

従来から厚生労働省は既存添加物の安全性確認を進めているが，本品目も国による安全性確認試験（反復経口投与毒性試験）を実施することになり，今回食品添加物として流通実績のある同一製品を試験対象試料として入手した．そこで，われわれは，既存添加物ウルシロウの品質規格および品質評価法設定の基礎資料とするために，この安全性

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試験対象試料のウルシロウ製品を用い，GC/MSによる脂肪酸組成の分析を行った．脂肪酸組成については，漆の生ろうおよび天日さらしろうでの報告[5]があるが，どのような組み合わせでトリグリセリドが構成されているかについての詳細な報告は見られない．そこでさらにLC/MSを用い，ウルシロウ中に含まれる各種トリグリセリドの分析条件を検討し，各トリグリセリドの結合脂肪酸種を明らかにするとともに，これらトリグリセリドの定量を行ったので併せて報告する．

実験方法

1. 試　料

ウルシロウ（英名：urushi wax）は，安全性試験の対象試料と同一ロットの1検体と，これと同じ製品で別ロットの1検体の計2検体を日本食品添加物協会を通じて入手した．今回入手したウルシロウ製品は，白～微黄色の粒状固体であった．業界自主規格（融点48～54℃，酸価30以下，けん化価200～235，ヨウ素価5～40）に適合した製品である．以下に示す分析は，この2検体につき，それぞれ3回の繰返し分析を行い，各分析値は2検体の平均値を求め，結果として示した．

2. 試　薬

トリグリセリド標品 (Table 1) として，glyceryl 1,2,3-tripalmitate (PPP) は Doosan Serdary Research Laboratories 社製，glyceryl 1,2-dipalmitate 3-oleate (PPO), glyceryl 1,3-dipalmitate 2-oleate (POP), glyceryl 1,2-dioleate 3-palmitate (POO), glyceryl 1,2-dipalmitate 3-stearate (PPS), glyceryl 1,2-distearate 3-palmitate (PSS), glyceryl 1-palmitate 2-oleate 3-stearate (POS) は Larodan Fine Chemicals AB 社製のものを使用した．パルミチン酸メチルエステル，ステアリン酸メチルエステル，オレイン酸メチルエステル，5% 塩化水素メタノール溶液は和光純薬工業(株)製，その他の溶媒はすべて市販特級品あるいはHPLC用を使用した．

3. 装　置

ガスクロマトグラフ/質量分析計：(株)島津製作所製 GC/MS system (GCMS-QP5050); 高速液体クロマトグラフ/質量分析計：Waters 社製 LC/MS system (LC: Alliance 2695; フォトダイオードアレイ検出器 (PDA): 2996 photodiode array detector; MS: Quatro Micro API).

Table 1. Structures of TG Standard Samples

Compound formula		MW	CH_2-R_1	CH-R_2	CH_2-R_3
PPP	$C_{51}H_{98}O_6$	807.3	P	P	P
PPO	$C_{53}H_{100}O_6$	833.3	P	P	O
POP	$C_{53}H_{100}O_6$	833.3	P	O	P
POO	$C_{55}H_{102}O_6$	859.3	P	O	O
PPS	$C_{53}H_{102}O_6$	835.3	P	P	S
PSS	$C_{55}H_{106}O_6$	863.4	P	S	S
POS	$C_{55}H_{104}O_6$	861.4	P	O	S

TG: triglyceride
Palmitic acid (P), Oleic acid (O), Stearic acid (S)

4. 構成脂肪酸の GC/MS 分析

ウルシロウ製品 6.75 mg を精密に量り採り，5% 塩化水素メタノール溶液 1.0 mL，ベンゼン3滴をスクリュー栓付き試験管内で混合し，沸騰水溶液中で3時間反応させた．冷却後水 1.0 mL を加え，ヘキサン（3 mL×2回）で抽出し，ヘキサン抽出液を約 1 mL に濃縮した．これにヘキサンを加えて 1.5 mL に調整した後，さらに10倍希釈したものを GC/MS 分析試料とした．別途各脂肪酸メチルエステル標品（パルミチン酸メチルエステル，ステアリン酸メチルエステル，オレイン酸メチルエステル）の 1.0 mg/mL ヘキサン溶液を調製し，標準溶液とした．次の操作条件で GC/MS 分析を行った．

GC/MS 測定条件：カラム，DB-1 (0.25 mm×30 m, 膜厚，0.25 μm, J&W Scientific)；カラム入口圧，100 kPa; キャリヤーガス流量，1.0 mL/min; 注入口温度，300℃; カラム温度，180℃→5℃/min→300℃ (8 min); イオン源温度，250℃; イオン化エネルギー，70 eV; 注入量，1.0 μL; 試料注入方式，スプリット (4 : 1); 測定モード，EI スキャン法 (m/z 50～800).

5. トリグリセリドの LC/MS および LC/MS/MS 分析

ウルシロウ製品をヘキサンに溶解し，濃度 5.0 μg/mL に調製したものを分析試料とし，Kusaka らの報告[6]を参考にして，次の条件で LC/MS 分析を行った．

LC/MS 条件：LC 条件：カラム，Capcell Pak C18 (4.6 mm i.d.×250 mm, 5 μm, Shiseido); 流速，0.4 mL/min; カラム温度，40℃; 移動相，アセトニトリル–ヘキサン–2-プロパノール＝60 : 0 : 40 (0 min)→40 : 20 : 40 (50 min)→40 : 20 : 40 (60 min); PDA, 192～600 nm; 検出波長，220 nm. MS 条件：ソース温度，130℃; 脱溶媒温度，400℃; 脱溶媒ガス流量，300 L/hr; コーンガス，50 L/hr; キャピラリー電圧，3.0 kV; コーン電圧，60 V (APCI pos.); スキャン範囲，m/z 100～1,000; SIM (Selected Ion Monitoring) $[M+Na]^+$. MS/MS 条件：コーン電圧，25 V (APCI pos.); コリジョンガス，アルゴン; コリジョン電圧，40V.

6. トリグリセリドの定量

ウルシロウ製品はヘキサンに溶解し，濃度 50 μg/mL に調製した．別に PPP (1 μg/mL), PPO (1 μg/mL), POO (0.1 μg/mL), POS (0.1 μg/mL), PPS (0.2 μg/mL) および PSS (0.1 μg/mL) を含む標準混合溶液を，ヘキサンを用いて調製した．これらを LC/MS に注入した．検出には選択イオン検出 (SIM) 法を採用し，次に示すトリグリセリド分子量関連イオンをモニターイオンとし，クロマトグラムピーク面積を求め，混合標準溶液で作成した検量線から各トリグリセリドを定量した．

PPP: m/z 829.6$[M_{PPP}+Na]^+$; PPO: m/z 855.6$[M_{PPO}+Na]^+$; POO: m/z 881.6$[M_{POO}+Na]^+$; POS: m/z 883.6$[M_{POS}+Na]^+$; PPS: m/z 857.6$[M_{PPS}+Na]^+$; PSS: m/z

885.6[M_{PSS}+Na]$^+$.

7. 異性体比の推定

LC/MS/MS 分析により，各トリグリセリド分子量関連イオンのプロダクトイオン m/z 551.3[PP]$^+$; m/z 577.3 [PO]$^+$; m/z 579.3[PS]$^-$; m/z 603.3[OO]$^+$; m/z 607.3 [SS]$^+$; m/z 605.3[OS]$^+$ の MRM (Multiple Reaction Monitoring) 測定を行い，トリグリセリドごとにプロダクトイオンのクロマトグラムピーク面積比を求め，標品で得られた値と比較することにより，異性体の存在および割合を推定した．

結果および考察

1. ウルシロウの構成脂肪酸の組成および相対含量の推定

ウルシロウ製品について TLC 分析（展開溶媒：ヘキサン–ジエチルエーテル–酢酸混液 (80 : 30 : 1)，検出：ヨウ素による発色）を行った結果，トリグリセリドを主成分とし，遊離脂肪酸やジグリセリドなどを少量含むことが明らかになった．

そこで，ウルシロウ製品の構成脂肪酸を確認するために，ウルシロウ製品をメタノリシスし，得られた脂肪酸メチルエステルを GC/MS で分析した (Fig. 1)．その結果，主成分としては保持時間 4.6 分（ピーク 1），5.9 分（ピーク 2），6.1 分（ピーク 3）の 3 つのピークが観察された．NIST ライブラリー検索および脂肪酸メチルエステル標品との比較の結果，ピーク 1 はパルミチン酸メチルエステル ($C_{17}H_{34}O_2$)，M$^+$: m/z 270，ピーク 2 はオレイン酸メチルエステル ($C_{19}H_{36}O_2$)，M$^+$: m/z 296，およびピーク 3 はステアリン酸メチルエステル ($C_{19}H_{38}O_2$)，M$^+$: m/z 298 と同定した．TIC クロマトグラム上に観察された総ピーク面積を 100% として各成分の相対含量を示すと，ピーク 1, 2, 3 は，それぞれ 72.5%, 17.1%, 8.8% であり，パルミチン酸メチルエステルの占める割合が非常に高かった．ほかに，保持時間 7.6 分（ピーク 4），10.2 分（ピーク 5）および 11.9 分（ピーク 6）に極小のピークが観察された．これらは，NIST ライブラリー検索を行った結果，ピーク 4 はアラキジン酸メチルエステル ($C_{21}H_{42}O_2$)，M$^+$: m/z 326，ピーク 5 はイコサン二酸ジメチルエステル ($C_{22}H_{42}O_4$)，[M－OCH_3]$^+$: m/z 339，ピーク 6 はドコサン二酸ジメチルエステル ($C_{24}H_{46}O_4$)，[M－OCH_3]$^+$: m/z 367 と推定された．相対含量は，それぞれ 0.6%, 0.8%, 0.2% で，これらの総量は全体の約 1.6% であり，ピーク 1, 2, 3 の含量と比較し非常に微量であった．したがって，今回入手したウルシロウ製品の構成脂肪酸は，パルミチン酸，オレイン酸およびステアリン酸を主とすることが示された．

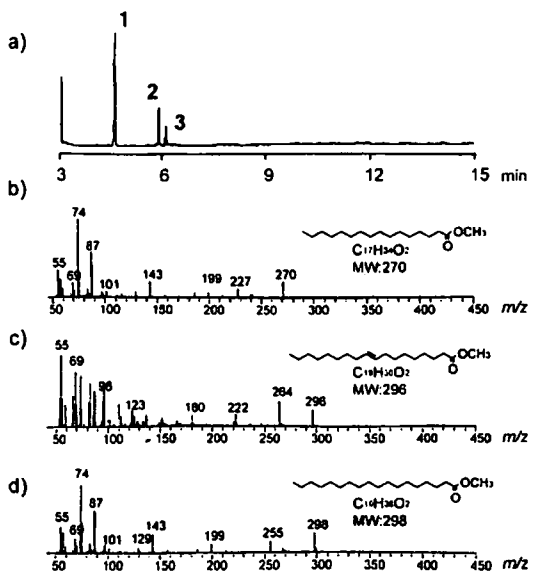

Fig. 1. GC/MS analysis of fatty acid methyl esters obtained by methanolysis of urushi wax

a) TIC, b) MS spectrum of peak 1, c) MS spectrum of peak 2, d) MS spectrum of peak 3

本研究では，安全性試験対象試料として入手可能であったウルシロウ製品 2 検体の脂肪酸組成について分析し，その平均値を求めた．これまでに，橘ら[5]は漆の生ろうと漂白のため天日さらししたものを用い，それぞれ分画して得た遊離脂肪酸画分とグリセリド画分の脂肪酸組成を調査しているが，ウルシロウの主要構成脂肪酸がパルミチン酸，オレイン酸およびステアリン酸である点は，本研究のウルシロウ製品の結果と一致した．しかし，実験材料と実験方法が異なるためか，脂肪酸の詳細な組成比や微量脂肪酸については，類似しているものの一致しない部分も見られた．したがって，既存添加物以外の目的で使用される漆ろうの脂肪酸組成が，今回の結果と類似しているか否かについては，別に検討を行う必要があると考える．

2. ウルシロウの構成成分および定量

次に，ウルシロウ製品中の各脂肪酸グリセリンエステルの構成脂肪酸の種類を明らかにするために，LC/MS 分析を行った (Fig. 2)．その結果，保持時間 44.55 分 (P-1), 45.48 分 (P-2), 46.43 分 (P-3), 49.27 分 (P-4), 50.22 分 (P-5), 53.87 分 (P-6) の 6 つのピークが観察された．P-3 は，APCI(pos.) において，m/z 829.7 を与え，これは，ウルシロウの主成分とされるグリセリンパルミタート (glyceryl tripalmitate, PPP (MW: 807.3)) の分子量関連イオン [M+Na]$^+$ に相当した．また同様に，P-1, 2, 4, 5, 6 は，それぞれ m/z 881.8, m/z 855.7, m/z 883.8, m/z 857.8, m/z 885.8 を与えた．メタノリシス後の GC/MS による分析の結果，ウルシロウ製品の構成脂肪酸が，主としてパルミチン酸 (P)，オレイン酸 (O)，ステアリン酸 (S) であったことを踏まえ，トリグリセリド標品 POO, PPO, PPP, POS, PPS および PSS と保持時間を比較した結果，ピーク P-1～6 とそれぞれ一致した．

しかし今回の LC 条件においては，glyceryl dipalmitate monooleate の異性体である PPO と POP の標品は同じ保持時間に溶出され，分離することができなかった．

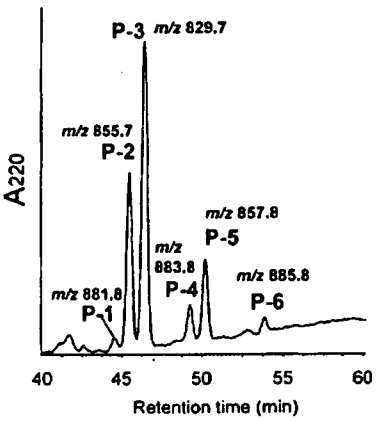

Fig. 2. HPLC chromatogram of urushi wax at UV 220 nm

The *m/z* values of the peaks show the pseudomolecular ions detected by LC/MS APCI(pos.) mode

Table 2. Possible TG Isomers in Urushi Wax by LC/MS APCI Mode

Peak No.	Observed [M+Na]$^+$	Predicted			
		Formula	TG isomers		
P-1	881.8	$C_{55}H_{102}O_6$	POO	OPO	
P-2	855.7	$C_{53}H_{100}O_6$	PPO	POP	
P-3	829.7	$C_{51}H_{98}O_6$	PPP		
P-4	883.8	$C_{55}H_{104}O_6$	POS	PSO	SPO
P-5	857.8	$C_{53}H_{102}O_6$	PPS	PSP	
P-6	885.8	$C_{55}H_{106}O_6$	PSS	SPS	

TG: triglyceride
[M+Na]$^+$: pseudomolecular ion

同様に，他のトリグリセリドについても，脂肪酸の結合位置が異なる異性体は，区別されずに検出されたと推測した．よって，ピーク P-1～6 は，脂肪酸の結合位置が異なるトリグリセリド異性体の混合物であると考えた (Table 2).

次に，今回の LC/MS 条件では，異性体である PPO と POP の標品は同じ分子イオン強度を与えたことから，他のトリグリセリド異性体についても同様であり，各ピークが異性体の混合物であったとしても，各トリグリセリドのいずれか 1 種の異性体を標品とすれば，SIM 法により定量可能と判断した．各種トリグリセリド標品 (PPP, PPO, POO, POS, PPS, PSS) を用い絶対検量線を作成し，定量を行った結果，ウルシロウ中に glyceryl tripalmitate (PPP) が 30.7±5.0%, glyceryl dipalmitate monooleate (PPO および POP) が 21.2±2.4%, glyceryl dioleate monopalmitate (POO および OPO) が 2.1±0.6%, glyceryl monopalmitate monooleate monostearate (POS, PSO および OPS) が 2.6±0.3%, glyceryl dipalmitate monostearate (PPS および PSP) が 5.6±0.9%, glyceryl distearate monopalmitate (PSS および SPS) が 1.4±0.3% (mean±SD, $n=4$) であり，PPP と PPO (PPO と POP の混合物) が主成分であることが分かった．

Laakso らは，トリグリセリドのマススペクトルのフラグメントパターンには異性体ごとに特異性があると指摘している[7,8]．そこで標品 PPO および POP を Product ion scan 法で比較した結果，プロダクトイオン $[M-RCOO]^+$ の相対強度に注目すると，PPO では $[PP]+/[PO]^+$ が 0.86 であるのに対し，POP では 0.46 であり，PPO と POP では明らかに異なっていた (Fig. 3a, b)．また，標品 PPO および POP を混合して，PPO の割合が，0, 25, 50, 75, 100% となるように調製した溶液のプロダクトイオン比 $[PP]+/[PO]^+$ を測定したところ，Fig. 4 に示すように直線関係が成立することが分かった．一方，ウルシロウ製品 2 ロットを用いてピーク P-2 (Fig. 3c) の $[PP]+/[PO]^+$ を調べたところ，その値は 0.769 および 0.772 であった．したがって P-2 は，PPO と POP の混合物のピークであ

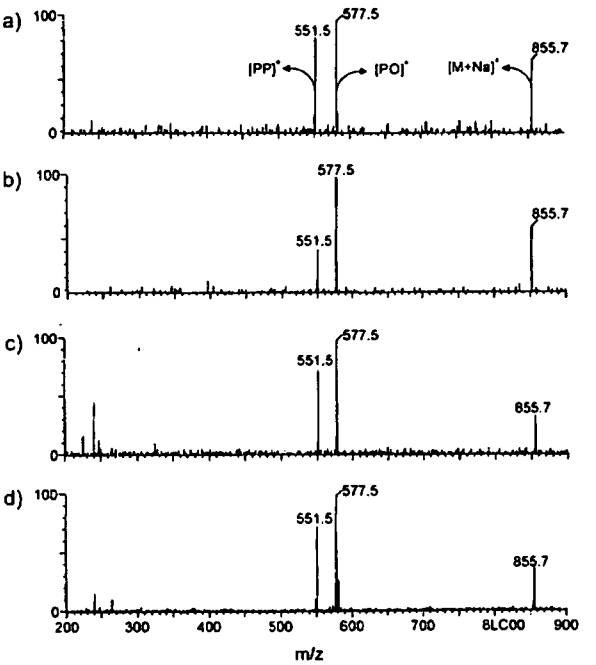

Fig. 3. LC/MS/MS spectra of urushi wax peak P-2 in Fig. 2 and triglyceride isomer standard samples

a) PPO; b) POP; c) urushi wax peak P-2 in Fig. 2; d) mixture of PPO/POP=78/22

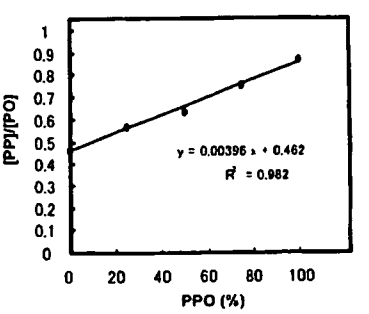

Fig. 4. Relationship between percentage of PPO in the mixture of PPO and POP isomers and product ion ratio of [PP]/[PO]

Table 3. Diacylglycerol Fragment Ions of TG Peaks in Urushi Wax and Their Ratios by LC/MS/MS

Peak No.	Predicted				[M−RCOO]⁺			Relative intensity ratios of [M−RCOO]⁺ ion peaks	
	Formula	TG isomers							
P-1	$C_{55}H_{102}O_6$	POO	OPO		[OO]⁺	[PO]⁺		[OO]⁺/[PO]⁺	0.37
P-2	$C_{53}H_{100}O_6$	PPO	POP		[PP]⁺	[PO]⁺		[PP]⁺/[PO]⁺	0.78
P-3	$C_{51}H_{98}O_6$	PPP			[PP]⁺				
P-4	$C_{55}H_{104}O_6$	POS	PSO	SPO	[PO]⁺	[PS]⁺	[OS]⁺	[PS]⁺/[PO]⁺	1.14
								[PS]⁺/[OS]⁺	1.38
								[OS]⁺/[PO]⁺	0.86
P-5	$C_{53}H_{102}O_6$	PPS	PSP		[PP]⁺	[PS]⁺		[PP]⁺/[PS]⁺	0.58
P-6	$C_{55}H_{106}O_6$	PSS	SPS		[SS]⁺	[PS]⁺		[SS]⁺/[PS]⁺	0.84

TG: triglyceride
[M−RCOO]⁺: diacylglycerol fragment ion created by the loss of a fatty acid group from the TG molecular ion.

Table 4. Diacylglycerol Fragment Ions of Authentic TGs and Their Ratios by LC/MS/MS

TG	[M−RCOO]⁺			Relative intensity ratio of [M−RCOO]⁺ ion peaks	
POO	[OO]⁺	[PO]⁺		[OO]⁺/[PO]⁺	0.53
PPO	[PP]⁺	[PO]⁺		[PP]⁺/[PO]⁺	0.86
POP	[PP]⁺	[PO]⁺		[PP]⁺/[PO]⁺	0.46
POS	[PO]⁺	[PS]⁺	[OS]⁺	[PS]⁺/[PO]⁺	0.60
				[PS]⁺/[OS]⁺	0.66
				[OS]⁺/[PO]⁺	0.91
PPS	[PP]⁺	[PS]⁺		[PP]⁺/[PS]⁺	0.74
PSS	[SS]⁺	[PS]⁺		[SS]⁺/[PS]⁺	0.57

TG: triglyceride
[M−RCOO]⁺: diacylglycerol fragment ion created by the loss of a fatty acid group from the TG molecular ion.

り，Fig. 4 の関係式から，PPO の割合が 2 ロットの平均値として 77.9% であることが予想された．実際に，標品 PPO と POP を 78 : 22 に混合したものは，ウルシロウ製品のピーク P-2 と同様な保持時間を示し，両者のピーク P-2 はほぼ同様なフラグメントパターンを与えた．したがって，ウルシロウ中の glyceryl dipalmitate monooleate は，PPO と POP が約 8 : 2 の割合で混在していると推測された (Fig. 3c, d)．ピーク P-1, 4〜6 についても，プロダクトイオンの相対比が標品 POO, POS, PPS, PSS と一致しなかったことから，それぞれ対応する異性体の混合物であることが予想された (Table 3, 4)．しかしながら，対応する異性体すべての標品を入手できなかったため，これらの異性体混合比については測定を行わなかった．

従来，トリグリセリド混合物に含まれる脂肪酸の結合位置が異なるトリグリセリド異性体を分別定量することは難しいとされてきた．トリグリセリドの脂肪酸を膵リパーゼを用いて位置特異的に加水分解し，得られた脂肪酸の組成比を測定することで，1, 3 位と 2 位の脂肪酸をそれぞれ分析することは行われてきたが，個々のトリグリセリド異性体を分別定量できる方法ではなかった．今回，LC/MS/MS 分析でプロダクトイオン [M−RCOO]⁺ の相対強度を測定することによりトリグリセリド異性体を分別定量できることを示した．

ま と め

われわれは，既存添加物ウルシロウの安全性確認試験の試験対象試料 1 製品（2 検体）の成分を確認するとともに，既存添加物ウルシロウの規格および品質評価法設定のための基礎的知見を得るため，ウルシロウ製品中の主成分の確認および定量を行った．ウルシロウ製品は，トリグリセリドを主成分とし，遊離脂肪酸やジグリセリドなどを少量含むことが確認された．GC/MS 分析の結果，ウルシロウの構成脂肪酸は，主としてパルミチン酸，オレイン酸，ステアリン酸であることが分かった．ウルシロウ製品中のトリグリセリドを，LC/MS-SIM 法によって定量した結果，主成分として glyceryl tripalmitate が 30.7±5.0%, glyceryl dipalmitate monooleate が 21.2±2.4% 含まれており，他に微量成分として，glyceryl dioleate monopalmitate が 2.1±0.6%, glyceryl monopalmitate monooleate monostearate が 2.6±0.3%, glyceryl dipalmitate monostearate が 5.6±0.9%, glyceryl distearate monopalmitate が 1.4±0.3% が含まれていることを明らかとした．以上のように，既存添加物ウルシロウの安全性確認試験対象試料の成分を明らかにすることができた．

また，LC/MS/MS 分析でプロダクトイオン比を比較することにより，脂肪酸の結合位置が異なるトリグリセリド異性体を分別定量できることを示した．

既存添加物の中には，ウルシロウのみならずいまだ国の品質規格の定められていないろう状物質が種々残されている．これらろう状物質品目間の判別が行えるよう，各添加物品目の成分組成に基づく品質規格作成を検討することが今後必要になると考えられる．既存添加物ウルシロウのトリグリセリド組成についてはこれまで報告されていなかったことから，本研究により得られたウルシロウ流通製品の詳細な成分解析結果は，今後の品質規格作成に有用であると考えられる．

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いたします.

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Quantitative nuclear magnetic resonance spectroscopic determination of the oxyethylene group content of polysorbates

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Abstract
Guidelines for the oxyethylene group (EO) content of polysorbates are set by the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Additives. However, the classical titration method for EO determination is difficult and time-consuming. Here, we show that quantitative ^{1}H-nuclear magnetic resonance spectroscopy can determine the EO contents of polysorbates rapidly and simply. The EO signals were identified through comparisons with sorbitan monolaurate and poly(ethylene glycol) distearate. Potassium hydrogen phthalate was used as an internal standard. The EO contents were estimated from the ratio of the signal intensities of EO to the internal standard. Two nuclear magnetic resonance systems were used to validate the proposed method. The EO content of commercial polysorbates 20, 60, 65, and 80 was determined to be within the recommended limits using this technique. Our approach thus represents an additional or alternative method of determining the EO contents of polysorbates.

Keywords: *Analytical method, food additive, oxyethylene, polysorbate, quantitative nuclear magnetic resonance*

Introduction

Polysorbates are non-ionic surfactants that are widely used as emulsifiers, dispersants, and stabilizers in food processing. Polysorbates consist of a mixture of fatty-acid partial esters of sorbitol and condensed sorbitol anhydrides, and contain approximately 20 moles of ethylene oxide (comprising the oxyethlene unit [EO] $-OC_2H_4-$) for each mole of sorbitol, along with its monohydrides and dianhydrides. The main fatty acids of polysorbates 20, 60, 65, and 80 are monolauric acid, monostearic acid, tristearic acid, and monooleic acid, respectively. The typical structures of these polysorbates are shown in Figure 1.

Guidelines for the EO contents of polysorbates are set by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA). To comply with the JECFA standards, the quality and composition of commercially synthesized polysorbates must be monitored and regulated. The standard method of measuring EO as described in "section VI. Methods for fats and related substances in the guide to specification" is as follows: "The oxyethylene groups are converted to ethylene and ethyl iodide which can be determined by titration. By utilizing a conversion factor determined on a reference sample, it is possible to compute the polyoxyethylene ester content" (JECFA [internet]). However, this classical titration method requires a complicated apparatus and involves several time-consuming steps. Alternative methods for determining the EO contents of polysorbates have not previously been reported, because these complex compounds are mixtures of isomers that are non-selectively substituted with EOs and fatty acids.

The quantitative nuclear magnetic resonance (qNMR) approach is based upon the International

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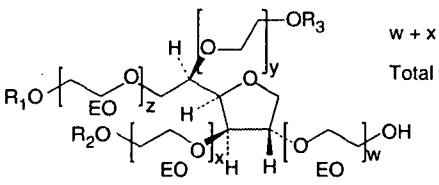


w + x + y + z = approx. 20

Total EO = (C_2H_4O) x (w + x + y + z) = $C_{40}H_{80}O_{20}$ (MW 881.1)

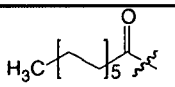
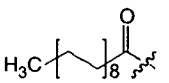
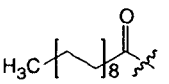
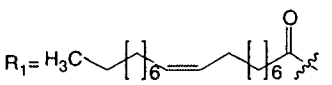
Compound			Formula (MW)	EO(%) in molecule
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	R_1= 	$R_2 = R_3 = H$	$C_{58}H_{114}O_{26}$ (MW1227.5)	EO(%) = 71.8
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	R_1= 	$R_2 = R_3 = H$	$C_{64}H_{126}O_{26}$ (MW1311.7)	EO(%) = 67.2
Polysorbate 65 (polyoxyethylene (20) sorbitan tristearate)	$R_1 = R_2 = R_3$ = 		$C_{100}H_{194}O_{28}$ (MW1844.6)	EO(%) = 47.8
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	R_1= 	$R_2 = R_3 = H$	$C_{63}H_{122}O_{26}$ (MW1295.6)	EO(%) = 68.0

Figure 1. Typical structures of polysorbates 20, 60, 65, and 80. The formulae and EO (%) were estimated based on the assumption that there were 20 moles of EO per molecule.

system of units (SI units). This valuable technique meets the requirements of a primary ratio analytical method (Jancke 1998). The use of qNMR to determine the ethanol content of deuterium oxide solution was previously reported as a part of an intercomparison study organized by the Comité Consultatif pour la Quantité de Matère (CCQM). The results showed that the accuracy of qNMR was equivalent to that of gas chromatography with a flame ionization detector (GC-FID) (Saito et al. 2003). qNMR exploits the fact that the signal intensities of a given NMR resonance are directly proportional to the molar amount of the nucleus within the sample. qNMR can determine the quantity of a compound, its substituent contents, or its absolute quality if the whole sample weight is known. This technique has several advantages for the analysis of organic compounds: it is non-destructive, it provides both quantitative data and structural information about a compound, and high-throughput spectral-acquisition instruments are commercially available. The main drawback of the qNMR approach is that manual spectral assignment is required; however, this can easily be rectified by applying current NMR technical experiments such as total correlated spectroscopy (TOCSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond coherence (HMBC), etc.

Based on these features of qNMR, we predicted that the method could be used to determine the EO contents of polysorbates. In the current paper, we detail the application of qNMR along with an internal standard for the direct determination of the EO contents of polysorbates.

Materials and methods

Materials

Samples of reagent-grade polysorbates 20, 60, 65, 80, and sorbitan monolaurate (Span 20) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Poly(ethylene glycol) distearate was purchased from Sigma-Aldrich Japan KK (Tokyo). Commercial samples of polysorbates were obtained from companies A–E via the Japan Food Additives Association. The NMR solvents, methanol-d_4 and acetone-d_6 with 0.03% tetramethylsilane (TMS), were purchased from Isotec Inc. (Miamisburg, OH). Potassium hydrogen phthalate (PHP), which was standard grade for volumetric analysis according to Japanese Industrial Standard (JIS) K8005, was purchased from Wako Pure Chemical Industries, Ltd.

Instrumentation

NMR spectra were recorded on JNM-ECA (500 MHz; JEOL, Tokyo) and MERCURY (400 MHz; VARIAN, Palo Alto, CA) pulsed Fourier-transform (FT) spectrometers, equipped with 5 mm $^1H\{X\}$ inverse detection gradient

Table I. Instruments and acquisition parameters.

Spectrometer	MERCURY400 (VARIAN) and ECA500 (JEOL)
Probe	5 mm indirect detection probe
Spectral width	2.5–12.5 ppm
Data points	64 000
Flip angle	45°
Pulse delay	30 s ($>5 * T_1$)
Scan times	8
Sample spin	15 Hz
Probe temperature	25°C
Solvent	Mixture of methanol-d_4 and acetone-d_6 (1:1)
Internal standard	Potassium hydrogen phthalate (PHP)
Range of integral signal	Oxyethylene group (EO) = 3.40–3.85 ppm 4 protons of PHP = 7.46–7.66 ppm + 8.18–8.38 ppm

probes, with methanol-d_4:acetone-d_6 (1:1) and 0.3% (w/v) PHP as an NMR solvent. The spectra were referenced internally to TMS by ^{1}H-NMR. The samples and internal standard were weighed on a LIBROR AEG-80SM (Shimadzu, Kyoto, Japan) electronic balance to an accuracy of ±0.01 mg.

Preparation of samples and NMR measurement conditions

The polysorbate samples were prepared as follows. PHP was crushed into a powder in a mortar and dried for 1 h at 120°C. After cooling in a desiccator, the powder (300 mg) was dissolved in 100 ml of methanol-d_4:acetone-d_6 (1:1) with ultrasonic agitation for 30 min. This stock solution was used as the NMR solvent and included an internal standard. A 50-mg polysorbate sample was then dissolved in 3 ml of the NMR solvent described above, and 0.6 ml of the sample solution was placed into a 5-mm NMR tube (Kusano Science Co. Ltd, Tokyo). The ^{1}H-NMR spectra were recorded on MERCURY400 and ECA500 spectrometers operating at 400 and 500 MHz, respectively. Typical ^{1}H-NMR parameters for the quantitative analyses are listed in Table I. The free induction decay (FID) signals of the samples from the MERCURY400 and ECA500 spectrometers were loaded onto a Windows XP-based personal computer (PC) equipped with the Alice 2 Version 5 (JEOL) NMR data-processing and analytical software. Fourier transformations of the FID signals were carried out with this software using the default parameters; window function = exponential, BF = 0.12 Hz, zero filling = 1, T1 = T2 = 0%, T3 = 90%, T4 = 100%. After phase adjustments and baseline corrections of the NMR spectra were performed using the same algorithms in the automatic mode of Alice 2, the signal intensities of the EOs and internal standard protons were measured, respectively.

Results and discussion

Identification of EO signals in polysorbates

Polysorbate molecules contain approximately 20 moles of EO according to the JECFA definition. However, recently reported matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra showed that polysorbates include numerous other chemical species, including polyethylenes, unesterified, monoesterified, and diesterified polyoxyethylene sorbitans, and isosorbides (Frison-Norrie and Sporns 2001). Furthermore, analysis by liquid chromatography (LC)–mass spectrometry (MS) confirmed that polysorbates contain not only polyoxyethylene sorbitan fatty acid esters but also numerous intermediates, such as polyoxyethylene sorbitan and isosorbitan, and the monoesters and diesters of fatty acids (Vu Dang et al. 2006). These studies have confirmed that polysorbates comprise many types of chemical isomers. This molecular diversity makes it difficult to determine the EO contents of polysorbates. However, we hypothesized that the EO contents of polysorbates could be measured rapidly and simply by qNMR if the signals could be identified on ^{1}H-NMR spectra, regardless of whether they contained numerous chemical isomers.

Thus, in order to identify the EO signals in polysorbates, we compared the ^{1}H-NMR spectra of polysorbate 20, sorbitan monolaurate, and poly(ethylene glycol) distearate. The partial structures of sorbitan monolaurate and poly(ethylene glycol) distearate, which comprised a sorbitol anhydride core and poly(ethylene glycol), were similar to those of polysorbate 20 (Figures 2 and 3). The sorbitan monolaurate and poly(ethylene glycol) distearate spectra revealed fatty-acid moiety signals with δ_H values ranging from 0.9 to 2.4 ppm, similar to those of polysorbate 20. The triplet signal at $\delta_H c.$ 0.9 ppm, the major broad signal and multiplet signal at $\delta_H c.$ 1.3 ppm and 1.6 ppm, and the triplet signal at $\delta_H c.$ 2.4 ppm were identified as the terminal CH_3–, $-CH_2-$, and $-CH_2C{=}O-$ groups of the fatty acids, respectively. Most of the EO signals in poly(ethylene glycol) distearate were observed between δ_H values of 3.40 and 3.85 ppm. One of the $-CH_2O-$ groups appeared to have been shifted downfield to $\delta_H c.$ 4.2 ppm, near to the residual proton of methanol-d_4 at $\delta_H c.$ 4.4 ppm. A HMBC experiment revealed that the proton at $\delta_H c.$ 4.2 ppm was correlated to the carbonyl carbon of the fatty acid at δ_C 173.4 ppm. Thus, the proton signal was assigned

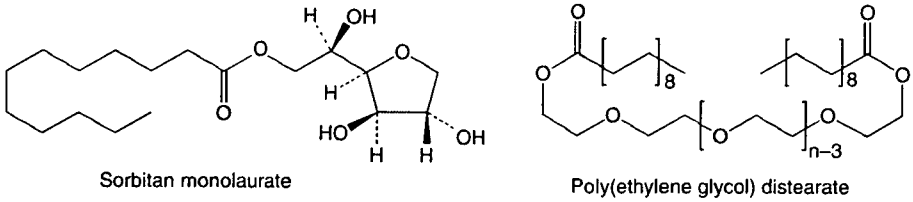


Figure 2. Structures of sorbitan monolaurate and poly(ethylene glycol) distearate.

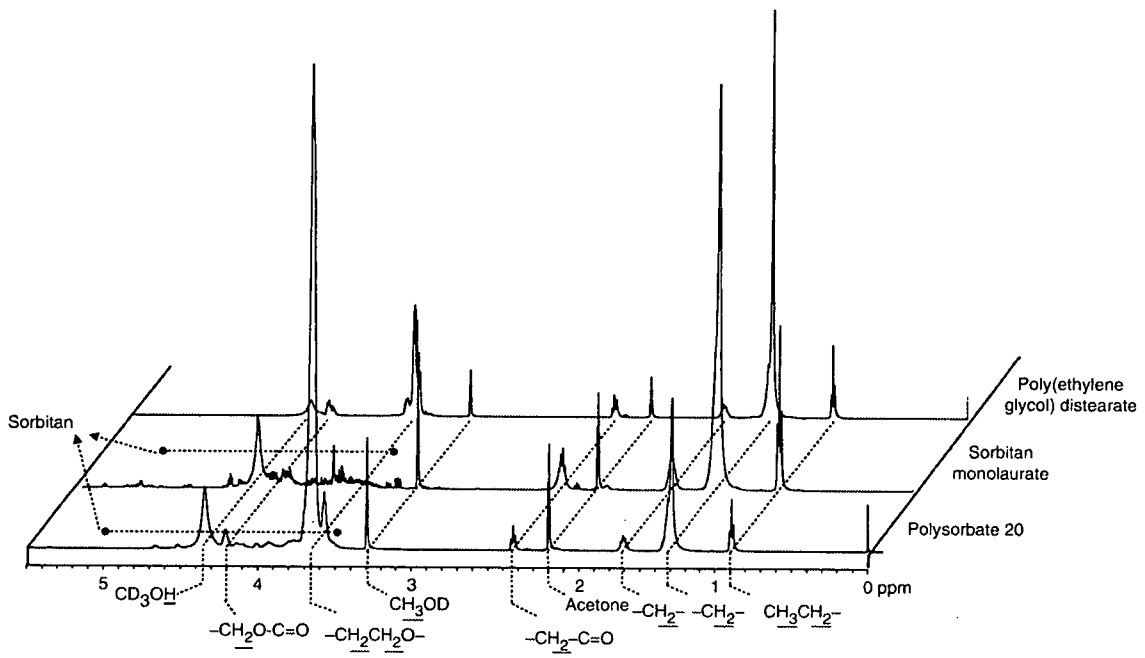


Figure 3. Comparison of NMR spectra of polysorbate 20, sorbitan monolaurate and poly(ethylene glycol) distearate. ^{1}H-NMR spectra were obtained using the ECA500 system (500 MHz; JEOL) under the conditions shown in Table I.

to the $-CH_2O-$ group adjacent to the fatty acid side chain. In the sorbitan monolaurate spectrum, various minor proton signals were observed from δ_H values of *c.* 3.4–5.0 ppm; these were attributed to the sorbitan moiety in sorbitan monolaurate, which consists of a mixture of cyclic sorbitol-derived ethers (such as sorbitan, isosorbite, and other isomers). These signals were also observed on the spectrum of polysorbate 20. However, the signals were broad and negligibly smaller than that of sorbitan monolaurate, as polysorbate 20 has the diversity of molecule more than sorbitan monolaurate. The polysorbate 20 signals ranging from δ_H 0.9 to 2.4 ppm that were attributed to the fatty-acid moiety were similar to those of sorbitan monostearate and poly(ethylene glycol) distearate. Polysorbate 60, 65, and 80 also showed the signals of fatty acid as same as sorbitan monolaurate, but the olefinic protons were only observed at δ_H 5.3 ppm on the spectrum of polysorbate 80 consisting of an unsaturated fatty acid (data not shown). The EO signals were assigned to a large envelop between δ_H 3.40 and 3.85 ppm, and at δ_H 4.20 ppm, which overlapped with the negligible small broad signals seen for the mixture of sorbitan, isosorbite, and other isomers moieties between δ_H values of *c.* 3.4 and 5.0 ppm. The EO signals of polysorbates 60, 65, and 80 also appeared within these ranges (data not shown). This was due to the fact that polysorbates basically comprise the same units: sorbitol anhydrides core, EO chains, and fatty acids. Although proton signals of the $-CH_2O-$ group adjacent to the fatty acid at δ_H *c.* 4.20 ppm were observed, and the signals of the sorbitol anhydrides core were overlapped on EO signals, they were negligible and did not effect the

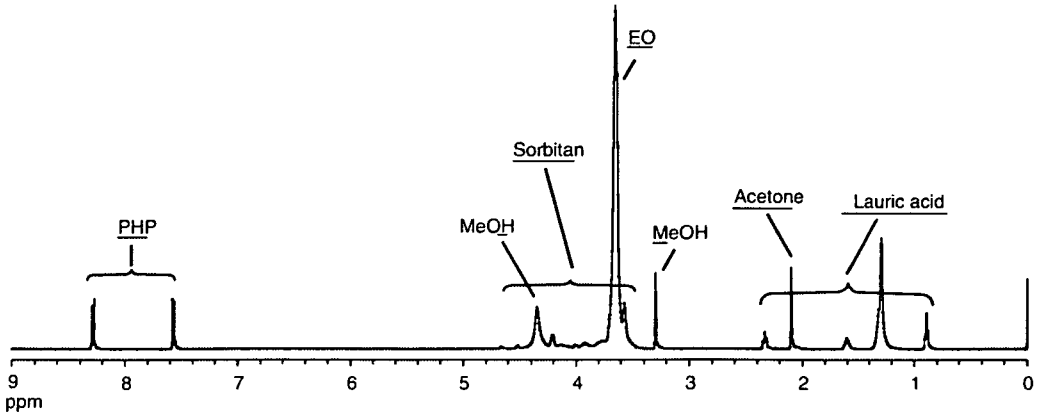


Figure 4. ^{1}H-NMR spectrum of polysorbate 20. The spectrum was obtained using the ECA500 system (500 MHz; JEOL). PHP was added as an internal standard. Signals of the four protons on the benzene ring of PHP were observed at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm. Most of the EO signals of polysorbate 20 were observed in a large envelope between δ_H 3.40 and 3.85 ppm.

determination of the EO contents. Thus, in the current research, we used the EO signals between δ_H 3.40 and 3.85 ppm to determine the EO contents of polysorbates by NMR.

Determination of EO contents in polysorbates 20, 60, 65, and 80

Several reports have described the applications of qNMR to determine specific types of chemical compound, such as natural products, impurities, and polymers (Stefanova et al. 1988; Paula 2001; Jake et al. 2002; Wells et al. 2002; Paula et al. 2005). Recently, a practical set of parameters for qNMR has been discussed (Saito et al. 2004). Furthermore, qNMR using an internal standard has been suggested as a new way of determining the contents of surfactants with a relatively high throughput (Koike et al. 2004a, 2004b, 2005). To minimize quantitative errors, we used the qNMR conditions described by Koike and colleagues, as listed in Table I. In particular, the flip angle was set to 45°, and the spectral width was set at a value sufficient for the peak of interest to fall within 80% of its centre, because the signal intensities decreased towards both edges of the spectral window. The number of data points was set at 64 000 to enhance the resolution. The pulse delay was set at up to 30 s, as high-precision NMR can only be achieved when the pulse delay time is greater than the quintuple spin-lattice relaxation time ($>5 * T_1$). As qNMR is based on the fact that the signal intensities of a given resonance are directly proportional to the molar quantity of the nucleus within the sample, the EO signal intensity of polysorbates and four protons on the benzene ring of PHP were used to determine the EO contents. The total time taken to obtain one FID using these parameters was <10 min.

The weight percentage of the EO groups was calculated according to Equation 1.

$$\mathrm{EO(w/w\%)} = \frac{(I_{EO}/H_{EO} \times M_{EO}/W_{sample})}{(I_{standard}/H_{standard} \times M_{standard}/W_{standard})} \times 100. \quad (1)$$

Here, I_{EO} is the signal intensity of the EO group; H_{EO} is the number of protons of the EO group (four); M_{EO} is the partial molecular weight of the EO group (44); W_{sample} is the weight (mg) of the sample in 3 ml of NMR solvent including PHP as an internal standard; $I_{standard}$ is the total signal intensity of PHP; $H_{standard}$ is the number of protons on the benzene ring of PHP (four); $M_{standard}$ is the molecular weight of PHP (204); and $W_{standard}$ is the weight (mg) of PHP in 3 ml of NMR solvent.

We initially confirmed that the qNMR showed linearity between the intensity of the EO signal and the amount of polysorbate 20. Various amounts of the reagent-grade polysorbate 20 sample were analysed by ^{1}H-NMR under the conditions described in the Materials and methods and Table I. The NMR spectrum of polysorbate 20 with the internal standard is shown in Figure 4. The four protons of the PHP benzene ring were observed as two double–doublet signals at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm, respectively. The ratio of the EO signal intensity was calculated as follows: intensity of EO/total intensities of four protons on PHP benzene ring. The relationship between EO/PHP and the amount of polysorbate 20 was linear ($R^2 = 0.9996$) in the range of 12.5–100 mg of polysorbate 20 in 3 ml of NMR solvent. Based on these results, we concluded

Table II. Determination of EO contents in polysorbates by qNMR.[a]

Sample name	MERCURY (400 MHz, VARIAN)			ECA500 (500 MHz, JEOL)		
	Entry	EO (%)	SD	Entry	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	1	73.0		1	72.2	
	2	71.8		2	71.8	
	3	73.2		3	72.3	
	4	71.7		4	72.5	
	5	71.9		5	71.6	
				6	72.9	
				7	72.0	
				8	72.7	
				9	73.7	
	AV	72.3	0.7	AV	72.4	0.6
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	1	67.7		1	67.4	
	2	65.3		2	67.7	
	3	68.9		3	67.5	
	4	67.8		4	67.9	
	5	66.9		5	68.6	
	AV	67.3	1.3	AV	67.8	0.5
Polysorbate 65 (polyoxyethylene(20) sorbitan tristearate)	1	49.1		1	49.8	
	2	49.8				
	3	49.5				
	4	49.8				
	5	48.7				
	AV	49.4	0.5			
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	1	65.0		1	67.0	
	2	65.5				
	3	66.2				
	4	64.8				
	5	65.1				
	AV	65.3	0.6			

[a]Reagent-grade polysorbates were purchased from Wako Pure Chemical Industries, Ltd. "Entry" means that the same sample was measured repeatedly on different days.

that qNMR could quantitatively determine the EO contents of polysorbates.

In order to verify whether qNMR could accurately determine the EO contents of polysorbates, two different NMR instruments (MERCURY and ECA500, with magnetic field strengths of 400 and 500 MHz, respectively) were used to repeatedly measure the EO contents of reagent grade polysorbates 20, 60, 65, and 80, which are generally used as standards. The results are shown in Table II. Reproducible results were obtained from each sample using the MERCURY system. Furthermore, the results obtained by the two NMR instruments did not differ significantly (standard deviations = 0.5–1.3%). These findings confirmed that it was possible to determine the EO contents of polysorbates using this approach regardless of the NMR instrument employed.

Finally, to confirm the validity of qNMR, we determined the EO contents of the commercially synthesized polysorbates 20, 60, 65, and 80, which met the specifications of the JECFA. All of the EO contents of the polysorbates were within the limits described in the *Compendium of Food Additive and Flavoring Agent Specifications* (JECFA [internet]) (Table III). The qNMR method for determining the EO contents of polysorbates demonstrated in this paper thus represents a simple and rapid alternative to the classic titration method recommended by the JECFA, which does not require specific chemical reactions or sophisticated apparatus. Moreover, the qNMR method made it possible to distinguish between Polysorbates 60 and 80, which have the same stipulated value, by comparison with the ^{1}H-NMR spectra, as polysorbate 80 consisting of an unsaturated fatty acid only showed the signals of olefinic protons at δ_H 5.3 ppm. It is theoretically possible to determine the ratio of a substituted group in any molecule, or the quality of any compound, using the proposed qNMR method with an internal standard, provided that the target proton signals can be separated from those

Table III. EO contents in commercial polysorbates determined using qNMR.[a]

Name	Stipulated value	Brand	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	70.0–74.0%	A	71.2	
		B	73.0	
		C	70.3	
		D	71.0	
		E	71.5	
		AV	71.4	1.0
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	65.0–69.5%	A	66.9	
		B	65.4	
		C	68.0	
		D	68.1	
		E	67.2	
		AV	67.1	1.1
Polysorbate 65 (polyoxyethylene (20) sorbitan tristearate)	46.0–50.0%	A	48.3	
		B	46.0	
		C	-	
		D	47.2	
		E	48.1	
		AV	47.4	1.1
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	65.0–69.5%	A	67.4	
		B	65.1	
		C	69.3	
		D	66.7	
		E	68.0	
		AV	67.1	1.6

[a]Brands A–E were purchased from five manufacturers. Brand C does not supply polysorbate 65.

of non-target groups and impurities. We are currently investigating the potential for this technique to determine various other compounds and polymers.

Conclusions

This research demonstrated that the EO contents of commercial polysorbates 20, 60, 65, and 80 could be readily determined using qNMR with an internal standard. Clear NMR data for the polysorbates were obtained from simple sample preparations. Two different NMR instruments validated the proposed method, and no significant differences were observed among the results. Moreover, the data obtained for commercial polysorbates 20, 60, 65, and 80 were in good agreement with the JECFA guidelines.

It is generally difficult to determine the amounts of substituted groups within polymers owing to their great diversity in molecular weights and structures. Classical methods require time-consuming preparation to set up the apparatus, and technically skilled operators. Furthermore, as there are no alternative methods to validate the results, they have to be accepted without verification. Our proposed qNMR is a rapid and simple analysis that provides the structural information of target compounds together. These advantages will reduce dramatically the time and manpower cost required, even if the NMR spectrometer and the solvents are expensive. qNMR is thus a valuable additional and/or alternative method, with a broad range of applications in quantitative analysis.

Acknowledgements

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Authentication and Chemical Study of Isodonis Herba and Isodonis Extracts

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Isodonis Herba is used as a Japanese dietary supplement and folk medicine. The extract of the herb (Isodonis extract) is also used as a food additive whose major compound is enmein (1). Here we compared internal transcribed spacer sequences of nuclear ribosomal DNA from Isodonis Herba available on the Japanese and Chinese crude drug markets, and found that the former derived from *Isodon japonicus* and *Isodon trichocarpus*, while the latter derived from distinct species such as *Isodon eriocalyx*. The liquid chromatography/mass spectrometry profiles of Isodonis Herba were classified into four chemotypes (A to D) according to the ratio of the major constituents. Types B and C contained 1 and oridonin (2) as major components, respectively. An intermediate (or mixed) form of types B and C in various ratios was designed type A. Type D contained eriocalyxin B (3) as its major component. Japanese herba were types A—C, while Chinese herba were types C and D. The commercial Isodonis extract products tested were classified as type D, suggesting that they originated from Chinese Herba. Understanding the relationship between extract constituents and DNA profiles is important for the official specification of dietary supplements and food additives of plant origin.

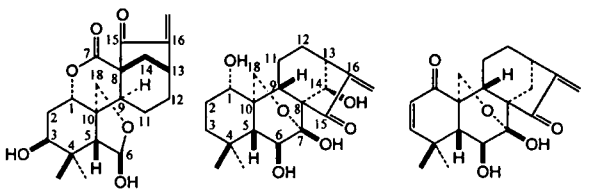
Key words Isodonis; LC/MS; internal transcribed spacer; folk medicine; food additive; dietary supplement

To ensure the quality of dietary supplements and food additives of plant origin, we have studied the main constituents of many products and identified the species from which they derive.[1—4] This work revealed a number of products that did not originate from the labeled material, including one-half of the commercial white kwao keur products purported to be made from the root of *Pueraria candollei* var. *mirifica*, two out of nine chondroitin sulfate products, and the commercial alkanet color, the major pigments of which were composed of shikonin and its derivatives rather than alkannin as labeled in Japan.

Isodon (previously *Rabdosia*) plants are widely distributed, and are the source of popular folk medicines in Japan and China. *Isodon japonicus* (Labiatae) is a perennial plant, which grows in Japan, Korea, eastern China, and far-eastern Russia.[5] In Japan, the aerial parts of *I. japonicus* and *Isodon trichocarpus* are used for the treatment of gastrointestinal disorders under the common name "enmei-so" (Isodonis Herba), which means "a grass for the prolongation of human life" in Japanese. "The Japanese Standard for Non-Pharmacopoeia Crude Drugs" defines these two species as the source plants of Isodonis Herba. In Japanese law system, Isodonis Herba are treated as "dietary supplements" and under the Japanese Food Sanitation Law when they are sold without the advertisement of their health effects. So, some dietary supplements utilizing the plants are sold in Japan, particularly in the form of herb tea. In China, other plants from this genus, such as *Isodon rubescens* and *Isodon eriocalyx*, are used as antibacterial and anti-inflammatory agents, and many studies have investigated the constituents and pharmacological activities of the former due to its anti-cancer activities.[6—10] As *Isodon* plants are rich in a wide range of diterpenoids, they are major targets of phytochemical studies.[11,12] The main constituents of *I. japonicus* include *ent*-6,7-seco kaurane, and *ent*-kaurane-type diterpenoids, such as enmein (**1**) and oridonin (**2**) (Fig. 1),[13—19] the antibacterial[20,21] and anti-inflammatory[22,23] activities of which have pharmacological effects against gastrointestinal disorders. In addition, some of the diterpenoids have an intensively bitter taste.[24]

The Isodonis extract from *I. japonicus* is also used as a natural food additive in Japan to give a bitter taste to processed foods, such as beverages, ice cream, and confectionery. "The List of Existing Food Additives" in Japan states that "Isodonis extract" is obtained from the stems or leaves of hiki-okoshi (*I. japonicus* HARA), and that the main bitter component is enmein (**1**).

On the other hand, some species of *Isodon* plants, collected and imported from various habitats and places are used as Isodonis Herba for a crude drug and a dietary supplement. In addition, it is possible to use these *Isodon* plants as the raw materials of the commercial Isodonis extracts. However, to our knowledge, there have been no previous investigations into the constituents and origins of Isodonis extract.



Enmein (1) Oridonin (2) Eriocalyxin B (3)

Fig. 1. Structures of *Isodon* Diterpenoids

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In the present study, the internal transcribed spacer (ITS) sequences of Isodonis Herba nuclear ribosomal DNA (rDNA) were analyzed to determine the original plant species from which they were derived. The major Herba diterpenoids were analyzed using liquid chromatography (LC)/mass spectrometry (MS). We then examined the relationship between the major constituents and the classification according to DNA analysis, and discussed the origin of Isodonis Herba and commercial Isodonis extract products.

Experimental

Materials and Reagents The commercial Isodonis extract product (Iso-E1) was obtained through the Japan Food Additives Association. Isodonis Herba samples (Iso-H1 to Iso-H8) were purchased from Japanese and Chinese local crude drug markets (Table 1). Authentic *I. japonicus* plants (Iso-1 to Iso-3) were provided by Japanese botanical gardens. *I. trichocarpus* (Iso-4 to Iso-6) and *I. shikokianus* var. *occidentalis* (Iso-7) plants were collected from the mountains in Ishikawa Prefecture and Toyama Prefecture, Japan (Table 2). Voucher specimens were deposited at our institute as herbarium specimens. For the LC/MS analysis, authentic **1** was purchased from Koshiro Company Ltd. (Osaka, Japan). All other chemical reagents were of research grade.

Instruments DNA sequencing analysis was performed using an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.) equipped with a 50-cm capillary array. The system was controlled by 3100/3100-Avant Data collection software, and the obtained electropherograms were analyzed using Applied Biosystems DNA sequencing analysis software version 5.1.

The LC/MS system (FractionLynx MS Autopurification System; Waters, Milford, MA, U.S.A.) consisted of a 2767 one-bed injection-collection sample manager, a 2525 binary high-pressure LC pump, a column/fluidic organizer (CFO), a 2996 photodiode array detector (PDA), and a ZQ single-quadropole mass spectrometer equipped with a Z-spray electrospray interface. The complete system was controlled by MassLynx software version 4.0. The electrospray sources ran at a 4.0-kV capillary voltage, 120 and 350 °C source and desolvation temperatures, respectively, and 350 and 50 l/h desolvation and cone gas flow rates, respectively. The cone voltage was 40 V. Full-scan acquisition between *m/z* 100 and 2000 was performed at a scan speed of 0.5 s/scan with a 0.1-s interscan delay. The solvent delivered to the electrospray interface was split in a 1 : 4 ratio, delivering to the interface at approximately 200 μl/min. The on-line PDA detector was monitored between 210 and 600 nm.

Nuclear magnetic resonance (NMR) spectra were recorded on the JEOL ECA-500 system (Jeol, Tokyo, Japan) in $CDCl_3$. The spectra were referenced internally to tetramethylsilane (TMS) in ^{1}H-NMR and to the solvent in ^{13}C-NMR. Assignment of the proton and carbon signals for all isolated compounds was confirmed by pulse-field gradient (PFG) ^{1}H–^{1}H correlation spectroscopy (COSY), PFG heteronuclear multiple quantum coherence (HMQC), and PFG heteronuclear multiple bond connectivity (HMBC) experiments.

DNA Sequencing Analysis of Isodonis Extract and Isodonis Herba A 20-mg sample of each product was frozen under liquid N_2 and crushed using a mixer mill, MM-300 (Qiagen, Hilden, Germany). Genomic DNA was extracted and purified from the powdered sample using a DNeasy Plant Mini Kit (Qiagen). The ITS region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) of the nuclear rDNA was PCR amplified using the obtained genomic DNA as a template. For the commercial samples except Iso-H4 to H6, nested PCR was used to compensate for low DNA yield. PCR was performed using a DNA engine PTC-200 (Bio-Rad, Hercules, CA, U.S.A.) with Gene TaqNT DNA polymerase (Nippon Gene, Tokyo, Japan) and the following program: 94 °C, 4 min; 40 cycles of 94 °C, 30 s, 50 °C, 30 s, and 72 °C, 45 s; then 72 °C, 4 min. The primers were designed based on the conserved sequence of the plant rDNA gene as follows: ITS-S1 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS-AS1 5′-TTTTCCTCCGCTTATTGATATGC-3′ for first-round PCR; and ITS-S2 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS-AS2 5′-GTAGTCCCGCCTGACCTG-3′ for second-round PCR. Excess primers and dNTPs were removed from the reaction mixture by Montage-PCR (Millipore, Billerica, MA, U.S.A.), and the amplicons were directly sequenced. Cycle sequencing was performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Subcloning of the amplicon into plasmid vectors was performed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The DNA sequences were aligned with a Clustal W program.[25] The genetic distances for all pairs of sequence were calculated using Kimura's two-parameter distance.[26] Neighbor-joining (NJ) tree[27] was constructed on the basis of their distances. The statistical support for the nodes of the tree was determined using bootstrap method[28] based on 10000 replicates.

Isolation of Oridonin (2) and Eriocalyxin B (3) MeOH extract (6.5 g) from 50 g Isodonis Herba (Iso-H2) was partitioned into hexane, $CHCl_3$, EtOAc, BuOH, and aqueous layers. The EtOAc portion (438 mg) was fractionated into six fractions by silica gel column chromatography with a gradient elution of hexane/acetone. The fourth fraction was subjected to reversed phase-high performance liquid chromatography (HPLC) equipped with YMC-Pack ODS-A (20×250 mm; YMC, Kyoto, Japan) with 45% MeOH isocratic elution, followed by treatment with activated charcoal and recrystallization from MeOH, affording 45 mg of **2**.

Oridonin (**2**): Colorless needles. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 1.14 (3H, s, Me-19), 1.30 (3H, s, Me-18), 1.36 (1H, m, H-3), 1.40 (1H, m, H-3), 1.48 (1H, d, J=6.9 Hz, H-5), 1.58 (1H, m, H-12), 1.84 (1H, m, H-2), 1.87 (1H, m, H-2), 1.93 (1H, m, H-9), 1.97 (1H, m, H-11), 2.43 (1H, m, H-12), 2.50 (1H, m, H-11), 3.21 (1H, d, J=9.2 Hz, H-13), 3.63 (1H, m, H-1), 4.28 (1H, dd, J=6.9, 10.3 Hz, H-6), 4.42 (1H, d, J=10.1 Hz, H-20), 4.80 (1H, d, J=10.1 Hz, H-20), 5.34 (1H, s, H-14), 5.50 (1H, s, H-17), 5.96 (1H, d, J=4.9 Hz, OH-1), 6.28 (1H, s, H-17), 6.94 (1H, d, J=10.3 Hz, OH-6). ^{13}C-NMR (125 MHz, pyridine-d_5) δ: 20.4 (C-11), 22.2 (C-19), 30.5 (C-2), 30.9

Table 1. Commercial Isodonis Extracts and Herba

Sample	Market	Collection distinct	Form
Iso-E1	Tokyo, Japan	Unknown	Extract[a]
Iso-H1	Osaka, Japan	China[b]	Herba
Iso-H2	Tokyo, Japan	Niigata Prefecture, Japan	Herba
Iso-H3	Osaka, Japan	Tokushima Prefecture, Japan	Herba
Iso-H4	Osaka, Japan	Niigata Prefecture, Japan	Herba
Iso-H5	Osaka, Japan	Niigata Prefecture, Japan	Herba
Iso-H6	China	Guizhou, China	Herba
Iso-H7	China	Henan, China	Herba
Iso-H8	China	Henan, China	Herba

a) Extract (Iso-E1) is a natural food additive used as a bittering agent in Japan. *b*) Herba was purchased from a Japanese market.

Table 2. Details of Authentic *Isodon* Plants Used in This Study

Sample	Species	Habitat	Voucher
Iso-1	*I. japonicus*	Tsukuba Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation (NIBIO), Japan	0548-79TS
Iso-2	*I. japonicus*	Tanegashima Division, Research Center for Medicinal Plant Resources, NIBIO	0068-99TN
Iso-3	*I. japonicus*	The Botanical Garden for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan	TM010
Iso-4	*I. trichocarpus*	Toyama city, Toyoma Prefecture, Japan[a]	TM011
Iso-5	*I. trichocarpus*	Kanazawa city, Ishikawa Prefecture, Japan[a]	TM007
Iso-6	*I. trichocarpus*	Kaga city, Ishikawa Prefecture, Japan[a]	TM008
Iso-7	*I. shikokianus* var. *occidentalis*	Kaga city, Ishikawa Prefecture, Japan[a]	TM009

a) Samples (Iso-4 to Iso-7) were wild plants, morphologically verified as authentic *Isodon* plants.

(C-12), 33.3 (C-18), 34.0 (C-4), 39.3 (C-3), 43.9 (C-13), 41.7 (C-10), 43.9 (C-13), 54.1 (C-9), 60.5 (C-5), 64.0 (C-20), 73.1 (C-1), 73.5 (C-14), 74.8 (C-6), 98.4 (C-7), 119.0 (C-17), 153.4 (C-16), 209.2 (C-15). ESI-MS (positive) *m/z* 387 $[M+Na\ (C_{20}H_{28}O_6Na)]^+$.

Commercial Isodonis extract (Iso-E1; 310 g) was partitioned into EtOAc, BuOH, and aqueous layers. The EtOAc portion (22 g) was fractionated into eight fractions using silica gel column chromatography with a gradient elution of $CHCl_3$/MeOH. The fourth fraction (4.2 g) was recrystallized from MeOH to give 1.8 g crystal composed of three compounds. The crystal (150 mg) was subjected to preparative thin-layer chromatography (TLC plate silica gel 60 F_{254}, 200×200×0.5 mm; Merck) with $CHCl_3$/MeOH (15/1), affording 80 mg of **3**.

Eriocalyxin B (**3**): Colorless powder. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.23 (3H, s, Me-19), 1.37 (3H, s, Me-18), 1.40 (1H, m, H-11), 1.50 (1H, m, H-12), 1.90 (1H, ddd, J=1.5, 4.9, 13.5 Hz, H-9), 2.04 (1H, d, J=8.8 Hz, H-5), 2.05 (1H, overlap, H-12), 2.14 (1H, ddd, J=1.0, 5.6, 12.6 Hz, H-14), 2.30 (1H, overlap, H-11), 2.33 (1H, brd, J=12.6 Hz, H-14), 3.46 (1H, brdd, J=4.6, 9.4 Hz, H-13), 3.95 (1H, dd, J=8.8, 12.0 Hz, H-6), 4.01 (1H, dd, J=1.7, 10.0 Hz, H-20), 4.29 (1H, dd, J=1.2, 10.0 Hz, H-20), 5.48 (1H, s, H-17), 5.81 (1H, d, J=12.0 Hz, OH-6), 5.85 (1H, d, J=10.0 Hz, H-2), 6.00 (1H, s, H-17), 6.77 (1H, d, J=10.0 Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 19.2 (C-12), 24.7 (C-14, 19), 29.8 (C-11), 30.0 (C-18), 34.3 (C-13), 35.9 (C-4), 46.6 (C-10), 48.3 (C-9), 56.9 (C-5), 59.7 (C-8), 65.6 (C-20), 73.1 (C-6), 95.5 (C-7), 119.0 (C-17), 127.1 (C-2), 152.5 (C-16), 161.3 (C-3), 196.9 (C-1), 208.3 (C-15). ESI-MS (positive) *m/z* 367 $[M+Na\ (C_{20}H_{24}O_5Na)]^+$.

LC-MS Analysis of Isodonis Extract and Isodonis Herba Samples (1 g) were extracted with 50 ml EtOH at room temperature for 24 h. The extract was passed through 5C filter paper (Advantec, Ehime, Japan) and then the filtrate was concentrated *in vacuo*. The residue was dissolved with 5 ml EtOH and the solution was filtered through a 0.45 μm Milex-LH membrane filter (Millipore). The filtrate (5 μl) was injected into the LC/MS system under the following conditions: column, YMC-J'sphere-ODS-H80 (4.6×250 mm; YMC); mobile phase, 40% MeOH (0 min) to 100% MeOH (30 min); flow rate, 1.0 ml/min; detection, ultraviolet (UV) 230 nm; and electrospray ionization (ESI) positive scan mode. The retention times of the three authentic compounds under the abovementioned conditions were as follows: 5.9 min for **1**, 13.3 min for **2**, and 17.2 min for **3**.

Results and Discussion

In the current study, we initially investigated the genetic diversity among commercial Isodonis Herba products available in crude drug markets using DNA sequence analysis. Genomic DNA was prepared from each sample, and the ITS1 region of the nuclear rDNA was used for sequence alignment analysis, as the ITS2 region could not be analyzed by direct sequencing in some samples. Multiple sequences of Iso-H1 were detected, so the Iso-H1 amplicon was subcloned into plasmid vectors and three clones were sequenced.

Authentic *I. japonicus* (Iso-1 to Iso-3), *I. trichocarpus* (Iso-4 to Iso-6), and *I. shikokianus* var. *occidentalis* (Iso-7) all had specific sequences in the ITS1 region, and no intraspecific mutations were detected. These three Japanese species could thus be differentiated on the basis of the ITS1 sequences, which were registered in international nucleotide sequence databases (the DNA DataBank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank) as follows: *I. japonicus*, AB292804; *I. trichocarpus*, AB292805; and *I. shikokianus* var. *occidentalis*, AB292806. The phylogenetic tree constructed from the ITS1 sequences of commercial Isodonis herba is shown in Fig. 2. All of the Isodonis Herba samples collected from Japan (Iso-H2 to Iso-H5) were estimated to originate from *I. japonicus* or *I. trichocarpus*, based on their ITS1 sequences. Among them, Iso-H2, Iso-H4, and Iso-H5 from Niigata Prefecture, Japan, derived from *I. trichocarpus*. This result is consistent with the distribution area of the plant on the Japan Sea side.[29] By contrast, the ITS sequences of Chinese Isodonis Herba (Iso-H1, and Iso-H6 to Iso-H8) differed from both *I. japonicus* and *I. trichocarpus*, so they were classified into other clusters. Furthermore, Iso-H1 had multiple sequences in the ITS1 region, although a single population was used in the genomic DNA preparation. This suggests that the source plant is a hybrid derived from varieties or species of *Isodon* plants. Based on these results, we concluded that the Chinese Isodonis Herba were distinct from, but closely related to, the original species of Japanese Isodonis Herba.

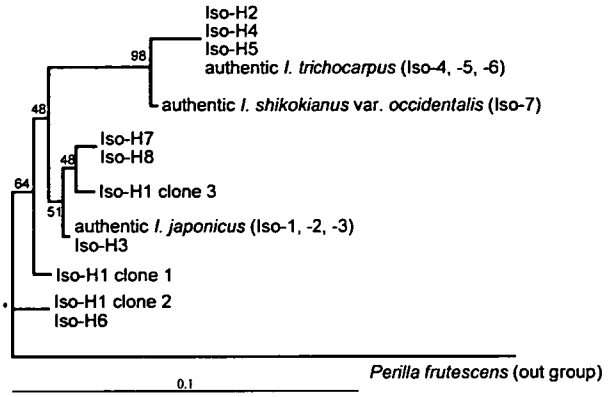

Fig. 2. Neighbor Joining Tree Constructed from ITS1 Sequences of Isodonis Herba

Bootstrap values in percent from 10000 replicates are indicated above the nodes. The tree is unrooted and branch lengths are proportional to the scale given in nucleotide substitution per site. *: trichotomy.

Subsequently, ethanol extracts prepared from authentic *Isodon* plants and Isodonis Herba were subjected to LC/MS analysis, in order to investigate the chemical diversity. The LC profiles at UV 230 nm differed according to the species and/or collection locations. Major chromatogram peaks were observed at retention time (RT) values of 5.9, 13.3, and 17.2 min, affording *m/z* 385 $[M+Na]^+$, 387 $[M+Na]^+$, and 367 $[M+Na]^+$ as the molecular related ions, respectively. The peak at RT 5.9 min was identified as **1** by comparison with the authentic sample. The peaks at RT 13.3 and 17.2 min were identified as **2** and **3**, respectively, after these constituents were isolated from Isodonis Herba and Isodonis extracts, and compared with reported ^{1}H- and ^{13}C-NMR spectra data.[30—33]

The profiles were classified into four chemotypes (A to D) based on the major constituents. Types B and C contained **1** and **2** as major components, respectively. The presence of an intermediate (or mixed) form of types B and C in various ratios was also observed, which was designated type A. Type D contained **3** as its major component, containing no traces of **1** or **2**. Typical LC profiles at 230 nm are shown in Fig. 3, and the results of the analysis of species and chemotype are summarized in Table 3. Among authentic *I. japonicus* (Iso-1 to Iso-3) and *I. trichocarpus* (Iso-4 to Iso-6), Iso-1 to Iso-5 contained both **1** and **2** while the predominant component varied according to the sample. Iso-6 mainly contained **2** and lacked **1**. Thus, authentic *I. japonicus* (Iso-1 to Iso-3) and *I. trichocarpus* (Iso-4 to Iso-6) were classified as type A, B, or C. By contrast, no peaks corresponded to any of the three compounds (**1**—**3**) in authentic *I. shikokianus* var. *occidentalis* (Iso-7), which showed an unidentified peak at an RT of 15.5 min as its main component.

Japanese Isodonis Herba (Iso-H2 to Iso-H5) contained

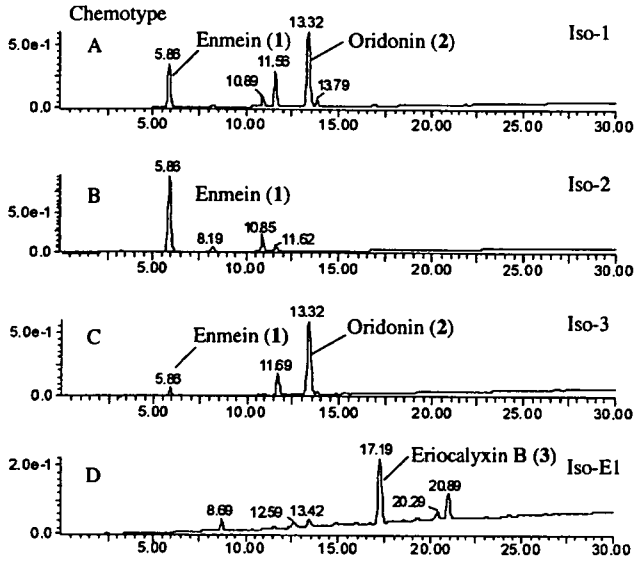

Fig. 3. Typical LC Profiles of Ethanol Extracts Prepared from Isodonis Extract and Isodonis Herba

The extracts were classified into chemotypes A, B, C and D by the major constituents: enmein (1) and oridonin (2) for A, 1 for B, 2 for C, eriocalyxin B (3) for D. The LC/PDA/MS conditions were described in experimental section.

Table 3. LC-PDA/MS Analysis of Authentic *Isodon* Plants and Isodonis Products

Sample	Species	Chemotype
Iso-1	***I. japonicus***	**A**
Iso-2	***I. japonicus***	**B**
Iso-3	***I. japonicus***	**A**
Iso-4	***I. trichocarpus***	**C**
Iso-5	***I. trichocarpus***	**B**
Iso-6	***I. trichocarpus***	**C**
Iso-7	***I. shikokianus* var. *occidentalis***	**Other**[a]
Iso-E1	Unknown	D
Iso-H1	Unknown	D
Iso-H2	Putative *I. trichocarpus*	C
Iso-H3	Putative *I. japonicus*	A
Iso-H4	Putative *I. trichocarpus*	C
Iso-H5	Putative *I. trichocarpus*	A
Iso-H6	Unknown	D
Iso-H7	Unknown	C
Iso-H8	Unknown	C

Authentic plants are indicated in the bold letter. *a)* No peaks corresponding to enmein (1), oridonin (2), or eriocalyxin B (3) were observed.

mainly **1** and/or **2**, and were classified as type A or C. Type B was not detected, even though it was predicted based on the results of authentic *I. japonicus* and *I. trichocarpus* in Japan. All Japanese Isodonis Herba contained **1**, while this was only weakly detected in Iso-H2 and Iso-H4. Chinese Isodonis Herba, Iso-H1, and Iso-H6 contained **3**, but lacked **1**, and were classified as type D. Iso-H7 and Iso-H8 mainly contained **2** and were type C. The commercial Isodonis extract product (Iso-E1) contained only **3**, and lacked both **1** and **2**, so was classified as the same type as Chinese Isodonis Herba Iso-H1 and Iso-H6 (type D).

The sequence of Iso-H1 clone 2 was identical to that of Iso-H6 (Fig. 2), and both contained **3** as their main component. This compound was first isolated from *I. eriocalyx*,[32] which was distributed in Yunnan, Guizhou, and Sichuan provinces in China, and was used as an antibacterial and anti-inflammatory agent in Yunnan province under the name "Yanshukang". Taken together, these facts and our results indicate that the Iso-H6 purchased from the crude drug market in Guizhou province was derived from *I. eriocalyx*.

Our results showed that the Japanese Isodonis Herba products (Iso-H2 to Iso-H5) originated from *I. japonicus* and *I. trichocarpus*, while the Chinese Isodonis Herba products (Iso-H1, and Iso-H6 to Iso-H8) originated from distinct species, such as *I. eriocalyx*. Furthermore, one (Iso-H1) of the Isodonis Herba products purchased in a Japanese market was made from other Chinese *Isodon* plants rather than *I. japonicus* and *I. trichocarpus*, which are defined as the source plants in "the Japanese Standard for Non-Pharmacopoeia Crude Drugs". We deduced that the Isodonis extract product (Iso-E1), which can be processed from Isodonis Herba, was also not made from the stated source plant with **1** as its major component, but was from the incorrect species with **3** as its major one.

A qualitative theory on the relationship between bitterness and the chemical structures of bitter *Isodon* diterpenoids has previously been proposed.[24] According to this theory, it is necessary for a bitter compound to contain at least one "unit" of bitter taste, which consists of a proton-donor group (PD) and a proton-acceptor group (PA) that must be within a distance of about 1.5 Å, thereby making it possible to form an intramolecular hydrogen bond. In the paper,[24] **1** and **2** were regarded as bitter compounds based on a qualitative test. Another paper[33] reported **3** as a bitter agent under the name, rabdosianone I. Furthermore, the structures of **1**, **2**, and **3** found in Isodonis Herba from Japan and China match the above criteria. Namely, the 6-aldehyde and 18-hydroxyl groups equilibrated with the acetal ring in **1** serve as PA and PD, respectively. The 6-hydroxyl and 15-carbonyl groups in **2** and **3** work as PD and PA, respectively. These facts indicate that the compounds are bitter. Therefore, the extract made from Chinese *Isodon* plants could also be used as a bitter agent.

In recent years, it has been reported that the bitter taste perception is involved with the G protein-coupled receptors (GPCRs) called T2Rs or TRBs.[34] Whether the response of the GPCRs against the *Isodon* diterpenoids relates to the above theory is of current interest.

Based on the results of the present study, we suggest that the definition of Isodonis extract in "the List of Existing Food Additives" in Japan should not be restricted to *I. japonicus* and that it should be expanded to *Isodon* plants. In addition, we recommend that the description of its main bitter components should also be changed to *ent*-6,7-seco-kaurane or *ent*-kaurane-type diterpenoids from enmein.

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Neocrocin A: a Novel Crocetin Glycoside with a Unique System for Binding Sugars Isolated from Gardenia Yellow

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A novel crocetin glycosyl ester, neocrocin A (2), was isolated from gardenia yellow. The structure of 2 was elucidated as that of an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl diester based on chemical and spectral data. The findings provide evidence that the binding system of crocetin glycosides is not limited to the anomeric position.

Key words *Gardenia jasminoides*; gardenia yellow; neocrocin A; crocetin glycoside; crocin

Crocin (**1**)[2)] is a digentiobiosyl all-*trans*-crocetin (8,8′-diapocarotene-8,8′-dioic acid) ester that is the major yellow pigment in gardenia yellow and saffron, which are extracts of *Gardenia jasminoides* fruits and *Crocus sativus* stigmas, respectively.[3,4)] Gardenia yellow and saffron consist of many minor pigments as well as some relatively abundant pigments which have been characterized as all-*trans*- and 13-*cis*-crocetin monoglucosyl ester,[2)] diglucosyl ester,[2)] monogentiobiosyl ester,[2,5)] glucosyl gentiobiosyl ester[2)] and gentiobiosyl neapolitanosyl ester.[5)] However, the structures of the other minor pigments have so far remained uncertain, and the binding systems for sugars have not previously been confirmed by detailed spectroscopic investigations, such as NMR analysis, after isolation. Here we report on the isolation and structural elucidation of a novel crocetin glycoside, neocrocin A (**2**), which has a unique binding system for sugars, based on spectral data and chemical derivatization (Fig. 1).

Results and Discussion

Gardenia yellow extracted from dried gardenia fruits was fractionated on a Diaion HP-20 column. The 60—70% methanol eluate was then concentrated and the residue was loaded into a preparative LC-MS system,[6)] led to the isolation of crocin (**1**) and neocrocin A (**2**).

Neocrocin A (**2**) was isolated as a red amorphous powder. The molecular formula of **2** was established as $C_{44}H_{64}O_{24}$, which was as the same as that of **1**, according to HR-ESI-MS (*m/z* 999.3707, $[M+Na]^+$, Calcd 999.3685), and the IR spectrum and UV/Vis absorption were similar to those of **1**. All-*trans*-crocetin dimethyl ester (**3**) and D-glucose were obtained, respectively, after the methanolysis and hydrolysis[7)] of **2**. These observations implied that **2** had the same carotenoid moiety, all-*trans*-crocetin, as the chromophore group, but that the binding system for glucoses differed from that of **1**. The ^{1}H- and ^{13}C-NMR spectra of **1** showed simple subduple signals, because **1** had C_2 structural symmetry. The ^{1}H-NMR spectra of **2** were similar to, but more complex than, those of **1**. Based on this comparison, we predicted that the C_2 structural symmetry was disrupted in **2** by the different binding system for glucoses at each end of crocetin, and that equilibrated isomerization could occur readily in the NMR solvent. The ^{1}H-NMR spectrum of **2** revealed a crocetin moiety (δ_H 6.49—7.31), anomeric doublets of β configuration (δ_H 5.38 (d, J=7.8 Hz), 4.53 (d, J=6.9 Hz), 4.13 (d, J=7.8 Hz), 4.15 (d, J=7.8 Hz)) and an anomeric doublet of α configuration (δ_H 5.06 (d, J=3.6 Hz)). Two anomeric protons (δ_H 4.53, 5.06) were shifted to a high magnetic field, and two oxymethines on H-2 (δ_H 4.39 (dd, J=3.7, 10.1 Hz), 4.53 (t, J=6.9 Hz)) were shifted to a low magnetic field, in comparison to those of **1**. This observation indicated the existence of a free hydroxyl group at an anomeric position on the glycosyl groups. Furthermore, HMBC correlations were observed between the H-2 of the α-glucoside and β-glucoside (glucose C) at δ_H 5.06 and δ_H 4.53, and the carbonyl carbons on the crocetin moiety at δ_C 167.92 and δ_C 167.11, respectively. Based on these chemical and spectral data, **2** was determined to be an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl ester. The NMR spectral data for **1** and **2** are summarized in Table 1.

Furthermore, to confirm the binding system for the sugars of neocrocin A (**2**), we firstly carried out peracylation of **2**. However, the sufficient quantity of peracetylated **2** was not obtained for the structure determination, because **2** was unstable more than crocin (**1**). Hence, after peracetylation of gardenia yellow, we isolated peracetylated crocin (**1a**) and an isomer of neocrocin A (**2a**). The other isomer of peracetylated **2** could not be isolated using preparative LC-MS because of overlapping with the peak of **1a**. The HR-ESI-MS spectra indicated that the molecular formula of both **1a** and **2a** was $C_{72}H_{92}O_{38}$. To compare the ^{1}H- and ^{13}C-NMR data between **2a** and **1a**, detailed 2D-NMR experiments were performed and the coupling constants were assigned using 1D-TOCSY. The chemical shifts and coupling constants of glu-

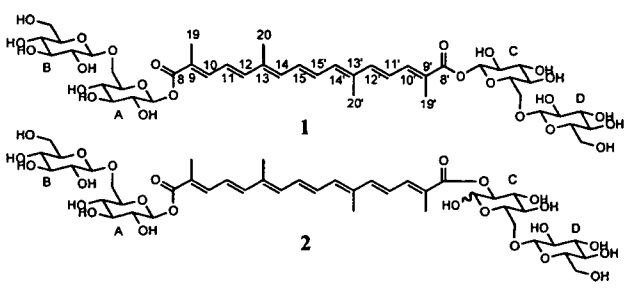

Fig. 1. Chemical Structures of Crocin (**1**) and Neocrocin A (**2**)

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