

が認められた。すなわち、市場流通品に比べ水耕栽培品の外部形態は、細長い円錐形、分枝が見られる、横切面が緻密で放射状に裂け目が見られない、などの特徴が認められ、また、内部形態は、道管径が小さい、シウ酸カルシウム単晶が多い、などの特徴が認められた。しかしながら、水耕栽培品の性状（外部形態及び内部形態）は、日局性状に合致すると判断できた。

径 0.5 cm 以上及び径 0.5 cm 未満に分別した根の検体各々について、グリチルリチン酸含量を測定した結果、クローンごとの平均値は径 0.5 cm 以上については、GuIV2 : 1.90%±0.15% (n=3)、GuIV1 系統 : 2.16%±0.89% (n=8) であり、径 0.5 cm 未満の検体については GuIV2 系統 : 1.55%±0.13% (n=3)、GuIV1 系統 : 1.90%±0.68% (n=8) であり、いずれの系統ともに日局不適であった。しかしながら個体ごとでは、GuIV1 の 2 個体（株 No. 4 及び株 No. 8）が日局規格値 (2.5%) に適合していた [GuIV1 株 No. 4 (径 0.5 cm 以上) : 2.89%、同 (径 0.5 cm 未満) : 2.57%、GuIV1 株 No. 8 (径 0.5 cm 以上) : 3.62%、同 (径 0.5 cm 未満) : 2.70%]。

さらに、グリチルリチン酸含量が日局規格に適合した 3 検体 (GuIV1 株 No. 4 径 0.5 cm 以上、同径 0.5 cm 未満、GuIV1 株 No. 8 径 0.5 cm 未満) について、日局理化学試験（確認試験、乾燥減量、灰分、酸不溶性灰分及びエキス含量の測定）を実施した結果、すべての項目において日局規格に適合した。

以上の結果から、甘草水耕栽培品は市場流通品（野生品）とは性状の細部が異なっているが、グリチルリチン酸含量が日局規格に適合したものに関しては、その他のすべての項目において日局規格に適合することが明らかとなつた。また、甘草水耕栽培品において、径 0.5 cm 以下の根であつても、短期間の栽培でグリチルリチン酸含量が日局規格を満たすものがあることは、水耕甘草の生薬としての調製法を検討する上で非常に興味深いと思われる。

#### 甘草市場流通品および水耕栽培品熱水抽出エキスの変異原性

甘草市場流通品 3 品および水耕栽培品 3 品より熱水抽出エキスを作製し、復帰突然変異試験 (Ames test) を行った結果、遺伝子突然変異誘発性は認められなかった。

#### 甘草市場流通品および水耕栽培品に含まれるヒ素および重金属

甘草市場流通品 4 品および水耕栽培品 3 品中に含まれるヒ素、鉛、カドミウム、水銀含量を ICP-MS を用いて定量した結果、市場流通品では、3 品からヒ素 (0.16-0.49 ppm)、1 品からカドミウム (0.13 ppm)、4 品すべてから鉛 (0.2-0.49 ppm) が定量された。一方、水耕栽培品のヒ素は検出限界以下、

カドミウム、水銀は定量下限以下であった。また鉛については市場流通品同様、すべての検体から定量値を得た (0.21-0.36 ppm)。いずれの甘草も、ヒ素、鉛に関しては日本薬局方、食品添加物公定書の基準値の 1/10 以下の濃度であったが、ヒ素とカドミウムに着目すると、水耕栽培品のほうがより安全性が高いことが確認された。

### 【まとめ】

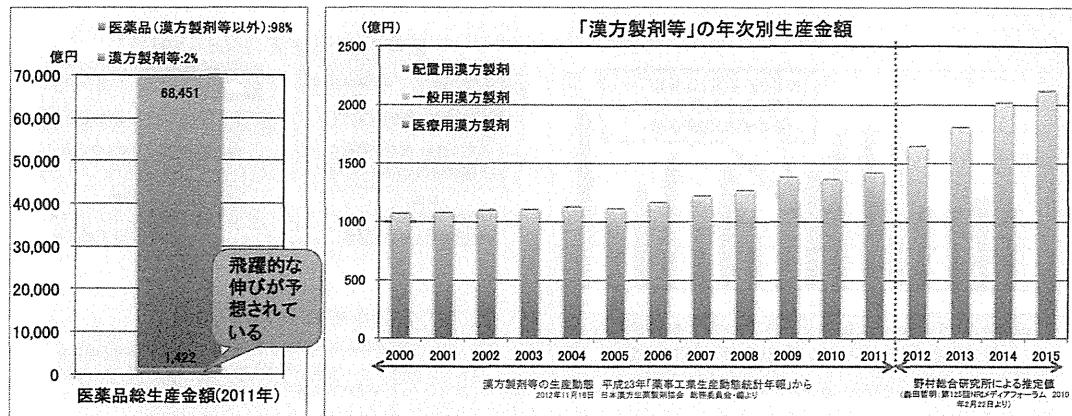
甘草の人工水耕栽培の確実な実用化を推進し、人工水耕栽培システムで生産した生薬への潜在的不安を解消するとともに、その有効性・安全性を担保するため、1)「甘草」等の種苗生産システムの構築、2) ハイテク「甘草」等生産システムの構築、3) 生薬「甘草」等の評価（安全性・有効性）に関する研究を実施した。その成果として、新規ウラルカンゾウ優良株育成法、新規優良株の育成、優良株識別法、新規水耕栽培装置の開発に成功した。安全性・有効性の評価に関しては、人工水耕栽培システムで生産した甘草は、野生植物由来の市場流通品と化学的に同等と考えられ、局方規格に適合すること、市場流通品と同様に変異原性は認められないこと、ヒ素及び重金属含量からは、市場流通品より安全性が高いと考えられることが判明した。

### 【謝辞】

本研究は、厚生労働科学研究費補助金（創薬基盤推進研究事業）「課題名：人工水耕栽培システムにより生産した甘草等漢方薬原料生薬の実用化に向けた実証的研究」により実施した。

### 【参考文献】

- 1) 吉松嘉代、河野徳昭、乾貴幸、千田浩隆、特願 2011-245757、「カンゾウ属植物株及びカンゾウ属植物増殖方法」(2011)
- 2) 吉松嘉代、河野徳昭、乾貴幸、特願 2013-049279、「カンゾウ属植物株、識別マーク、増殖方法、及び、水耕栽培装置」(2013)



- 超高齢社会の日本では生活習慣病、認知症等が増加し、漢方薬を処方される例が増加(2011年調査では漢方薬を処方する医師は89%、今後も増加する見通し)
- 2011年の漢方製剤等生産金額は1,422億円であるが、2015年には2000億円を超えると予想されている
- 漢方製剤等生産金額の81.9%(2011年)は医療用(薬価があり、原材料費の高騰を直ぐには回収できない)
- 漢方製剤等生産金額は、医薬品総生産金額の2%であるが、使用量が少ないわけではない(抗がん剤に比べ安価)

国産可能な生薬は多く、過去には輸出していた生薬もある(例:黄連)

しかし、国産生薬は輸入品に比べ価格が高い→手間がかかる、食糧に比べると需要が少ない→国内生産の衰退

図 1. 医薬品総生産高に占める漢方製剤等の生産高と漢方製剤等の年次別生産高



生薬を国内で確保・供給できる画期的技術が必要

図 2. 「甘草」を取り巻く状況

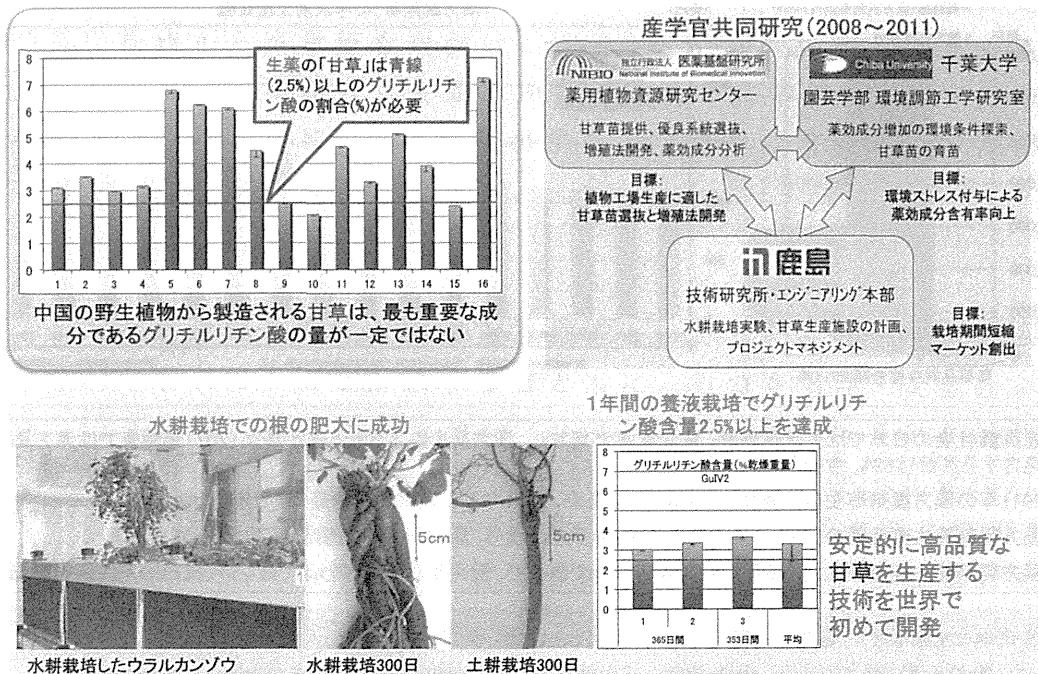


図3. 薬用植物（甘草）の人工水耕栽培の開発  
第9回産学官連携功労者表彰（厚生労働大臣賞）

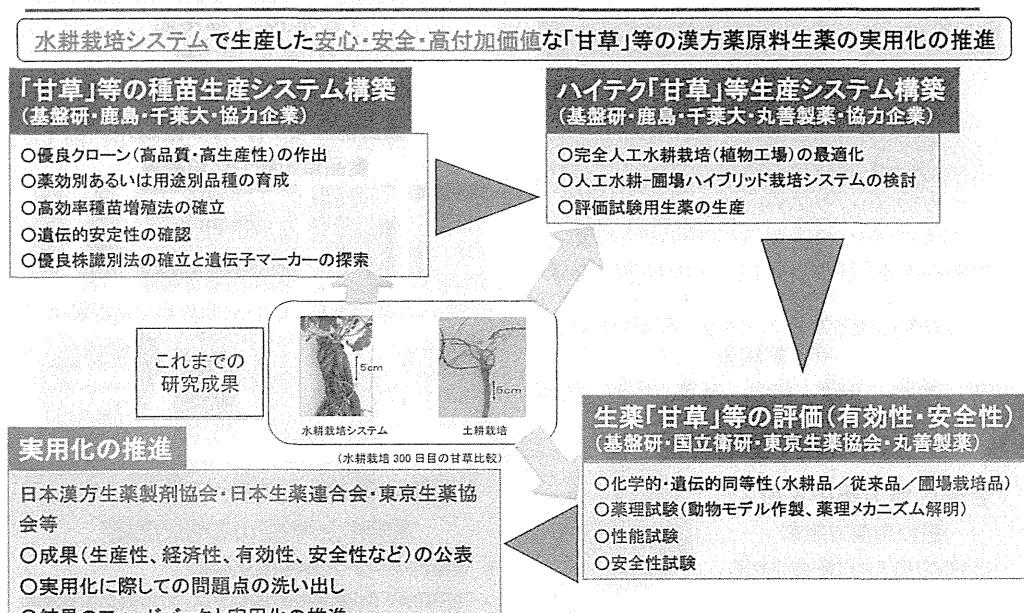


図4. 人工水耕栽培システムにより生産した甘草等漢方薬原料生薬の実用化に向けた実証的研究（H24-創薬総合-一般-007）

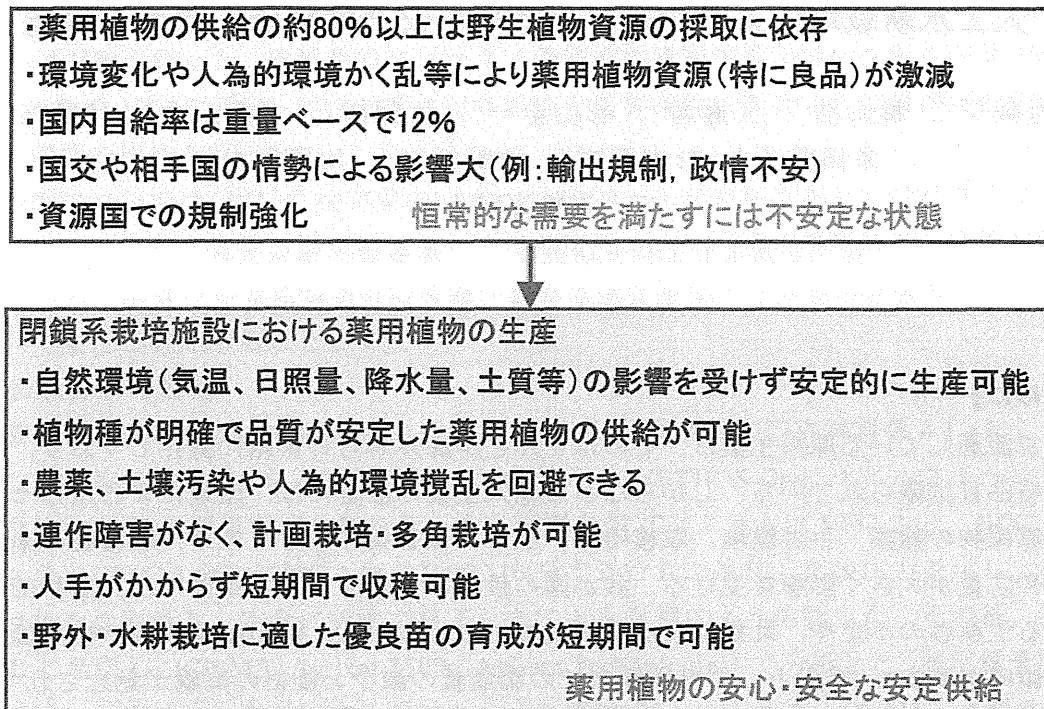


図 5. 人工水耕栽培システムによる薬用植物(生薬)の生産の背景と利点

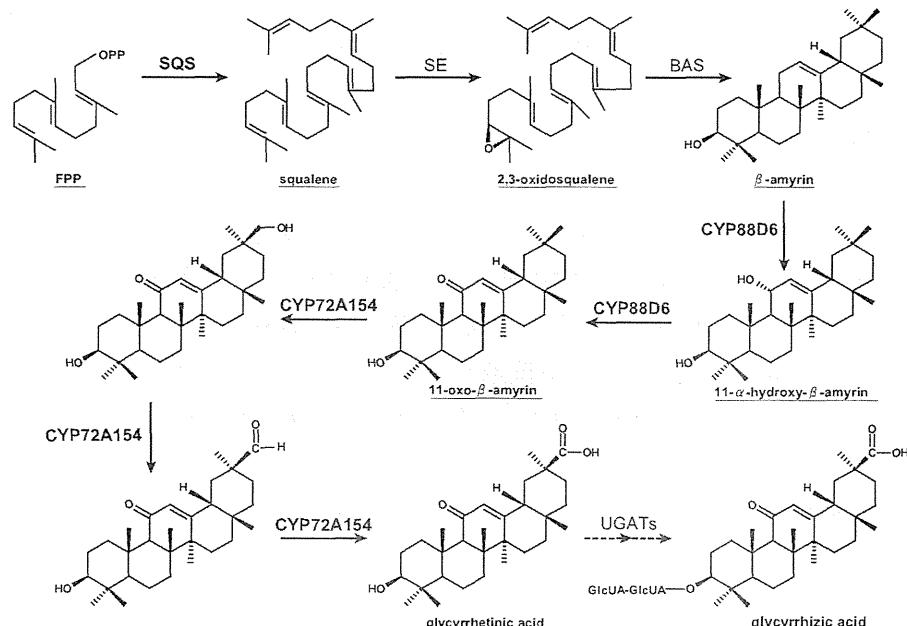


図 6. カンゾウ属植物におけるグリチルリチン酸合成経路

## 人工水耕栽培により生産した甘草の安全性評価に関する研究

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### 【はじめに】

国内で流通している原料生薬は、そのほとんどが海外からの供給に依存しており、国内供給できる品目は限られている。しかしながら輸入生薬の品質には、産地によるばらつきが生じ、基原植物の種類、生育環境、収穫時期、加工・保管条件などにより、収穫物の薬用成分の組成や含量が大きく影響を受ける。我が国最大の供給源である中国では、農村部の高齢化による生産者の不足や、乱獲による環境破壊への対策から、輸出総量規制や輸出価格の値上げの傾向が高まり、結果として日本国内での流通量の減少と価格の高騰が懸念されている。このような背景の中、我々は漢方薬原料生薬で最も需要が高く、国内流通のほぼ100%を輸入に依存している甘草について、人工栽培環境下での養液栽培により、短期間で安定的に生薬を生産する手法を国内で初めて開発した。

本研究では、水耕栽培システムで生産した甘草の安全性について、市場流通品と比較し、その同等性を評価する目的で、遺伝子突然変異誘発性試験（Ames試験）と4種の有毒微量元素（ヒ素および鉛、水銀、カドミウム）の定量を行ったので報告する。

### 【材料および方法】

#### 1. 甘草試料

市場流通品および水耕栽培品（鹿島建設株式会社での水耕栽培）由来の甘草熱水抽出エキス、あるいは粉碎試料は（独）医薬基盤研究所より提供を受けた。市場流通品3品、NIB-003、NIB-074、NIB-176および、水耕栽培品3品、GuIV2③-43、GuIV2⑥-2、GuIV2⑥-13の計6品の熱水抽出エキスをAmes試験に、市場流通品4品（前述の3品に加えNIB-007）および、水耕栽培品3品（前述と同じ）の粉碎試料を有毒微量元素の定量に用いた。

#### 2. Ames試験

Ames試験は試験菌株として、ヒスチジン要求性の *Salmonella typhimurium* 変異株 TA100、TA1535、TA98、TA1537およびトリプトファン要求性の *Escherichia coli* 変異株 WP2 *uvrA* の計5菌株を用いた。試験前日に解凍し37°Cで一晩振とう培養後、甘草6品の熱水抽出エキスにつき、各濃度（78.1-5,000 μg/plate）で試験を行った。ラット肝臓由来代謝活性化酵素（S9 mix）の存在下あるいは非存在下で、熱水抽出エキスと試験菌液を混和し、37°Cで20分間プレインキュベーションを行った後、低ヒスチジンあるいは低トリプトファン含有の軟寒天を加え、寒天平板培地にて37°Cで培養し、48時間後の復帰変異コロニー数を計測した。エキ

スによる直接的な菌株への生育阻害が認められた場合は、さらに低濃度のエキス (9.77-156  $\mu\text{g}/\text{plate}$ ) で試験を行った。陰性対照にはエキス調製液の希釀溶媒 (DMSO あるいは 70% エタノール) のみを 100  $\mu\text{L}$  用い、陽性対照には各菌株と培養条件に適合した変異誘導物質を用いて上記と同様の操作を行った。

判定基準については、用いた検定菌のいずれかの S9 mix 非存在下あるいは存在下において、各熱水抽出エキスを含有する平板培地上における変異コロニー数の平均値が、陰性対照の 2 倍以上に増加し、かつ、その増加に用量依存性あるいは再現性が認められた場合に当該試験系において陽性と判定することとした。

### 3. 有毒微量元素の定量

有毒微量元素の定量は、市場流通品 4 品と、水耕栽培品 3 品の粉碎試料各 0.1 g に、60% 硝酸 5 mL、35% 塩酸 500  $\mu\text{L}$  を添加し、マイクロウェーブ(ETHOS One, マイルストーンゼネラル株式会社)による密閉系湿式分解を行った。試料分解プログラムは 50°C 2 分、30°C 3 分、200°C 13 分、170°C 1 分、200°C 4 分、200°C 10 分で遂行し、冷却した後圧力を戻し、再度同プログラムを繰り返し、完全に灰化が終了したことを目視により確認した。その後、超純水で 50 mL に希釀し、ICP/MS (Agilent 7500c, Agilent Technologies) で分析した。測定質量数( $m/z$ ) については、干渉イオンを排除する目的から、ヒ素は 75、カドミウムは 111、水銀は 201、鉛は 208 を選択し、ヒ素のみコリジョンモード (ヘリウム) で測定した。すべての過程で用いた器具および容器は 2 mol/L の硝酸で洗浄し、超純水で置換した後に乾燥させたものを使用した。

## 【結果および考察】

### 1. Ames 試験による遺伝子突然変異誘発性の同等性評価

本研究において用いたいずれの検定菌においても、陰性対照と比較し 2 倍以上の復帰コロニー数の増加が陽性対照物質処理により確認されたことから、当該試験系は妥当であると判断した。試験の結果、市場流通品 3 品及び水耕栽培品 3 品すべてに関して、陰性対照のコロニー数の 2 倍以上の増殖を示した菌株は存在せず、遺伝子突然変異誘発性は認められなかった(図 1)。甘草熱水抽出エキスによる菌株への生育阻害が観察された試験では、さらに低濃度のエキスで再評価を行った。本来、グリチルリチン精製物はグラム陽性菌、陰性菌とともに抗菌試験では陰性と評価されているが<sup>1)</sup>、熱水抽出エキスを用いた検討で生育阻害が観察されたことから、エキスに含まれるフラボノイド画分等より由来する抗菌活性がある種の菌株に対して発現したものと想定された。しかしながら低濃度における再評価により、遺伝子突然変異誘発性は陰性と判断されたことより当該試料における安全性の同等性が示された。

### 2. 有毒微量元素の定量による同等性評価

まず予備試験として市場流通品を粉碎し、0.1 g を秤量した後に 60% 硝酸 5 mL で分解し、ICP/MS により 4 元素 (ヒ素および鉛、水銀、カドミウム) の分析を行った(表 1)。また回収率を指標に試料の調製法と測定法の確立の検討を行った。有毒微量元素の含有量が最も低い試料 NIB-007 に、ヒ素、カドミウム、水銀および鉛をそれぞれ 2  $\mu\text{g}/\text{g}$  添加して添加回収試

験を行ったところ、全ての金属において良好な回収率を得た（表2）。以降は確立した方法を用いて市場流通品4品と水耕栽培品3品の分析を行った。結果として、水銀は7品すべてが定量下限（0.1 μg/g）以下であった。カドミウムは市場流通品1品から0.13 μg/g、ヒ素は市場流通品3品から0.16~0.36 μg/g検出されたが、水耕栽培品からはいずれも検出されなかつた。一方、鉛については市場流通品および水耕栽培品ともにすべての検体から検出された。含有量は市場流通品で0.20~0.49 μg/g、水耕栽培品で0.21~0.36 μg/gであり、二群間における有意差はなかつた( $p=0.396$ )。しかしながらいずれの含有量も生薬規格における基準値の1/20以下であった（表3）。

### 【まとめ】

遺伝子突然変異誘発性に関しては、市場流通品3品および水耕栽培品3品ともにすべて陰性であり、両群に差はみられなかつた。有毒微量元素の分析より、半閉鎖的な環境で育成する水耕栽培品は、従来栽培法あるいは野生種の甘草と比較して、有毒微量元素の混入する危険性が低く、より安全性の高い生薬原料を生産できる可能性が示された。現在、水耕栽培品の有効性を検討するために、生薬甘草の薬理効果の中で、炎症性アレルギー疾患への抑制作用に着目した評価系を構築し、市場流通品および水耕栽培品の有効性に関する同等性の評価について実施している。

### 【謝辞】

本研究は厚生労働科学研究費補助金（創薬基盤推進研究事業）「課題名：人工水耕栽培システムにより生産した甘草等漢方薬原料生薬の実用化に向けた実証的研究」により実施した。

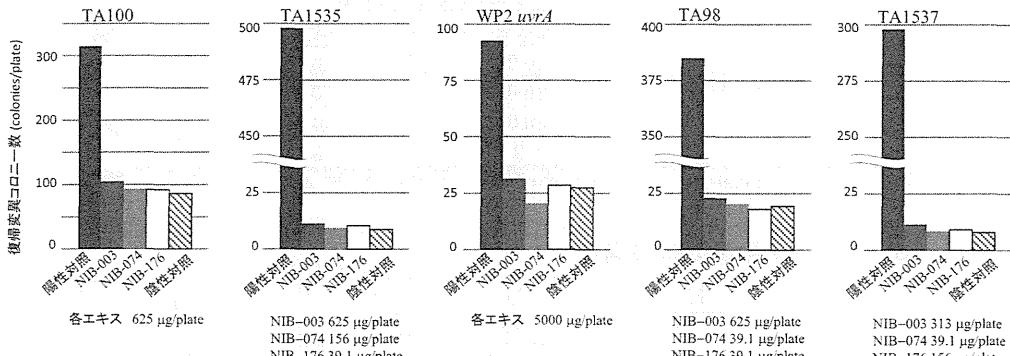
### 【参考文献】

<sup>1)</sup>農林水産省、マストリチン動物用医薬品製造承認申請書添付資料：薬理試験

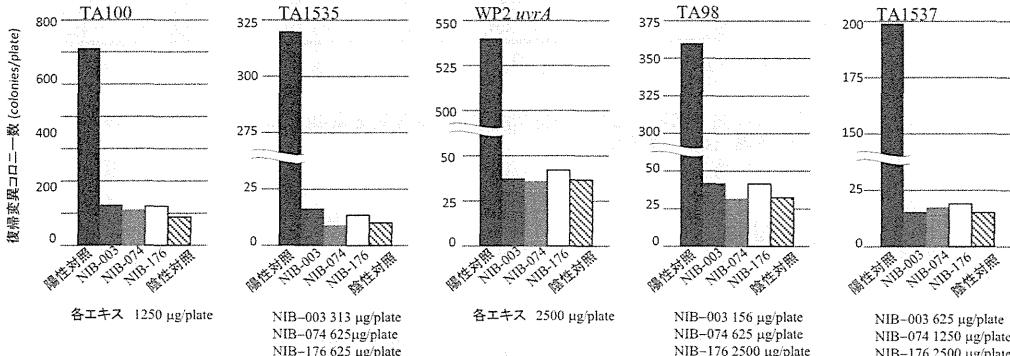
[図1] Ames試験による遺伝子突然変異誘発性に関する検討

## A. 市場流通品甘草

1) S9 mix (-)

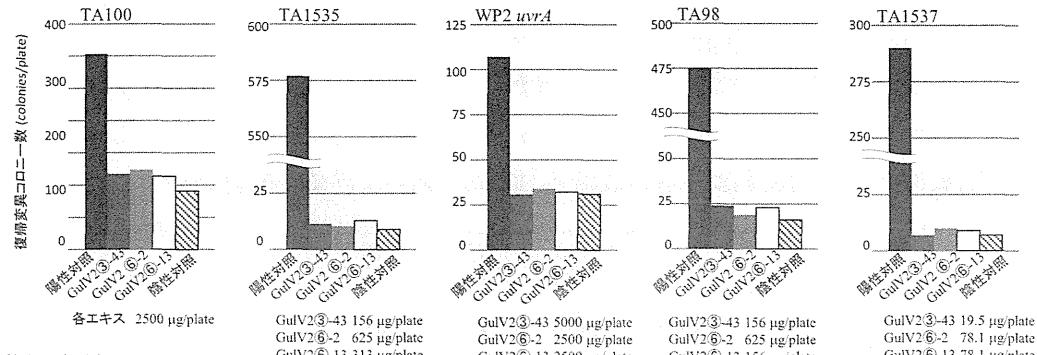


2) S9 mix (+)

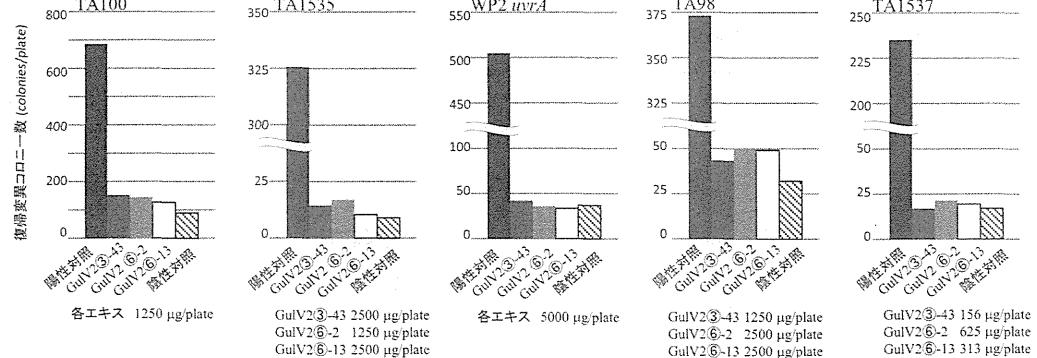


## B. 水耕栽培品甘草

1) S9 mix (-)



2) S9 mix (+)



[表1] 予備試験結果（添加回収試験用試料の決定）

## A. 試験溶液中の各同位体の濃度

	試験溶液濃度 (ng/mL)				
	<sup>75</sup> As	<sup>111</sup> Cd	<sup>200</sup> Hg	<sup>202</sup> Hg	<sup>208</sup> Pb
NIB-003	0.9	(0.0)	0.33	0.36	1.01
NIB-007	0.3	(0.0)	0.24	0.25	0.30
NIB-074	0.3	(0.0)	0.20	0.24	0.27
NIB-176	0.3	0.2	0.20	0.23	0.31
LOQ (ng/mL) <sup>a)</sup>	0.4	0.4	0.4	0.4	1

## B. 材質中の各同位体の濃度

材質重量(g)	材質中濃度 (μg/g)				
	<sup>75</sup> As	<sup>111</sup> Cd	<sup>200</sup> Hg	<sup>202</sup> Hg	<sup>208</sup> Pb
NIB-003	0.13257	0.4	< LOD	< LOQ	< LOQ
NIB-007	0.11806	< LOQ	< LOD	< LOQ	< LOQ
NIB-074	0.10199	< LOQ	< LOD	< LOQ	< LOQ
NIB-176	0.10148	< LOQ	< LOQ	< LOQ	< LOQ
LOQ (μg/g)		0.2	0.2	0.2	0.5

材質中濃度 (μg/g) = 試験用液濃度 (ng/mL) × 50 mL / 材質重量 (g) / 1000  
LOQ = 定量下限 LOD = 検出限界

[表2] 添加回収試験

Sample: NIB-007

	As <sup>a)</sup>	Cd	Hg	Pb
Recovery (%)	98.9	97.0	98.3	103.0
RSD (%)	3.7	1.9	2.7	4.2

全てのデータはn=5の平均値を示した

a) ヘリウムガスによるコリジョンモードでの測定

[表3] 市場流通品および水耕栽培品甘草のヒ素および重金属含有量

	(μg/g)			
	As	Cd	Hg	Pb
市場流通品				
NIB-007	< LOD	< LOQ	< LOQ	0.20 <sup>a)</sup>
NIB-003	0.36	< LOQ	< LOQ	0.49 <sup>a)</sup>
NIB-074	0.16	< LOQ	< LOD	0.28 <sup>a)</sup>
NIB-176	0.16	0.13	< LOD	0.28 <sup>a)</sup>
水耕栽培品				
GuV2-③-43	< LOD	< LOQ	< LOQ	0.36 <sup>b)</sup>
GuV2-⑥-13	< LOD	< LOQ	< LOQ	0.32 <sup>b)</sup>
GuV2-⑥-2	< LOD	< LOD	< LOD	0.21 <sup>b)</sup>
LOQ	0.1	0.1	0.1	0.05

すべての測定はn=1で行った

a)およびb)間に有意差なし(p=0.396)

LOQ = 定量下限 LOD = 検出限界

## Effects of Post-harvest Storage and Drying Temperatures on Four Medicinal Compounds in the Root of Chinese Licorice (*Glycyrrhiza uralensis*)

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The root of Chinese licorice (*Glycyrrhiza uralensis*) is used worldwide as a medicinal herb. The goal of this study was to understand changes in the concentrations and compositions of four medicinal compounds—glycyrrhizic acid (GL), liquiritin (LQ), liquiritigenin (LG), and isoliquiritigenin (ISLG)—in the root of Chinese licorice during post-harvest treatment. The effects of post-harvest storage temperatures ( $-80$ ,  $-30$ ,  $-13$ ,  $4$ , and  $25^{\circ}\text{C}$ ) and drying temperatures ( $30$ ,  $40$ ,  $50$ , and  $60^{\circ}\text{C}$ ) on concentrations of the four medicinal compounds were investigated. GL and LQ concentrations in roots stored at  $-30$  and  $-13^{\circ}\text{C}$  for 1–2 weeks tended to be 4% to 13% higher than GL and LQ concentrations in roots dried directly in a vacuum freeze dryer (controls). LG concentrations in roots stored at  $4^{\circ}\text{C}$  for 2 weeks were nearly 60-fold higher and ISLG concentrations at  $25^{\circ}\text{C}$  for 1 week were 10-fold higher than LG and ISLG concentrations in the controls. In addition, low temperature ( $30$  and  $40^{\circ}\text{C}$ ) drying compared to vacuum freeze drying (controls) increased LG and ISLG concentrations without decreasing GL and LQ concentrations. This study provided an approach to increase the target compound concentrations in Chinese licorice for different market demands (drugs, cosmetics, and food).

Keywords : flavonoids, glycyrrhizic acid, herbal medicine, secondary metabolites

### INTRODUCTION

Licorice (*Glycyrrhiza*) belongs to the family Leguminosae, and licorice root is the most common ingredient in traditional Japanese *Kampo* medicines. The principal pharmacologically active compound in licorice root is glycyrrhizic acid (GL). GL is a triterpenoid that has been shown to exhibit antiviral, immunomodulatory, and antitumorigenic activities (Pompeii et al., 1979; Chavali et al., 1987; Agarwal et al., 1991). Several studies also suggest that GL is highly active in inhibiting replication of the human immunodeficiency virus type 1 (HIV-1) and the severe acute respiratory syndrome (SARS)-associated virus (Hattori et al., 1989; Cinatl et al., 2003). According to the Japanese Pharmacopoeia (2011), licorice roots used in Japanese *Kampo* medicines are required to have GL concentrations above 2.5% dry weight (DW). GL is also a powerful natural sweetener (50–170 times sweeter than sucrose) used in food (Mukhopadhyay and Panja, 2008).

In recent years, liquiritin (LQ), liquiritigenin (LG), and isoliquiritigenin (ISLG) have been the major flavonoids studied in licorice roots. LQ from *Glycyrrhiza uralensis* has been reported to exert antidepressant-like effect in animal studies (Zhao et al., 2008). LG shows immunomodulatory and antioxidant activities (Pan et al., 2000; Lee et al., 2009). ISLG can be used as an oxidase in-

hibitor and shows anticancer activities (Pan et al., 2000; Kanazawa et al., 2003). Additionally, licorice root extracts are frequently used in cosmetics, because the flavonoids (including LQ, LG, ISLG, and others) have been shown to promote skin whitening and prevent skin pigmentation and aging (Wang et al., 2004), and as antioxidants added to foods including oil, ham, snacks, and instant noodles (You, 2001). Mixtures of compounds or single compounds from licorice root extracts are both used in different applications (Hayashi and Sudo, 2009; Zhao et al., 2010). Therefore, not only GL concentration but also concentrations of the other three major flavonoids should be controlled for various market demands (drugs, cosmetics, and food).

Licorice does not grow in the wild in Japan. Most licorices for the market demand were imported from other countries such as China, Afghanistan, and Turkmenistan (Hayashi and Sudo, 2009). However, with the increasing demands for licorice throughout the world market, wild licorices have been harvested excessively, leading to serious desertification of the grassland, and as a result, the exportation of wild licorice has been limited in these countries. Therefore, cultivated licorice is expected to increase as the principal source for licorice demands.

Until now, many studies concentrated on the effects of environmental factors and stress on the growth and GL concentration of Chinese licorice (*Glycyrrhiza uralensis*) (Afreen et al., 2005; Wang et al., 2008; Hou et al., 2010;

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Sun et al., 2012). Generally, reports show that the concentrations of secondary metabolites in harvested medicinal plants change during the storage and drying process (Du et al., 2004; Policegoudra and Aradhya, 2007). Little research has been conducted on the concentrations of medicinal compounds in Chinese licorice during the post-harvest process. Hayashi (2010) reported that GL concentrations increased in harvested Chinese licorice roots stored in the incubator at  $-5^{\circ}\text{C}$  for 4 weeks and in the storehouse at ambient temperatures ( $-15$  to  $8^{\circ}\text{C}$ ) for 8 weeks; however, the ambient temperatures ( $-15$  to  $8^{\circ}\text{C}$ ) were not controlled. Concentrations of LQ, LG, and ISLG as secondary metabolites in Chinese licorice root may change depending on storage temperature.

Although it is generally known that compound concentrations in medicinal plants dried in a vacuum freeze dryer can be steadily maintained, the cost of vacuum freeze drying is much higher than that of oven drying or natural air drying. Natural air drying is the most common method for drying medicinal plants during the production process because of the low cost, but drying time is long and drying temperature cannot be controlled. In contrast, oven drying offers short drying time and ease of temperature control. Therefore, oven drying was used in our study. Drying temperature can affect compound compositions in medicinal plants. Du et al. (2004) reported that increasing the drying temperature ( $40$ ,  $55$ , and  $70^{\circ}\text{C}$ ) of American ginseng (*Panax quinquefolium*) decreased the concentration of total ginsenosides but increased neutral ginsenosides and decreased malonyl ginsenosides. Therefore, the compositions of the four compounds in Chinese licorice root studied here may change depending on drying temperature.

To understand the changing concentrations and compositions of four medicinal compounds in Chinese licorice root during post-harvest treatment for different market demands, the effects of post-harvest storage and drying temperatures were investigated in this study.

## MATERIALS AND METHODS

### Plant materials and sampling method

Chinese licorice plants (2-month-old, line No. 2, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Japan) from cutting propagation were grown in a hydroponic system for 10 months under artificial environmental conditions. Main roots of the fresh Chinese licorice plants after harvest were separated from lateral roots and were cut into 1.0 to 1.5 cm lengths directly. Main root blocks (0.4–0.7 cm diameter) were collected from different Chinese licorice plants for use in the different treatments.

### Temperature treatments

#### Post-harvest storage temperature treatments

Root blocks were cut vertically into two parts (Fig. 1). One part was used as a control and was dried directly in a vacuum freeze dryer (FDU-1100, Tokyo Rikakikai Co., LTD., Japan) for 3 days. The other part was used in the treatments; treatment parts were stored at  $-80$ ,  $-30$ ,  $-13$ ,  $4$ , and  $25^{\circ}\text{C}$  for 1, 2, 3, and 4 weeks and then were

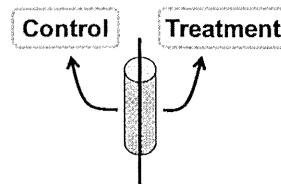


Fig. 1 Diagram of method used to cut root block into two pieces.

dried in a vacuum freeze dryer for 3 days after storage temperature treatment. Each storage temperature treatment consisted of 3–4 repetitions.

### Drying temperature treatments

Root blocks were cut vertically into two parts (Fig. 1). One part was used as a control and was dried directly in a vacuum freeze dryer for 3 days. The other part was used in the treatments; treatment parts were dried in the oven at different drying temperatures ( $30$ ,  $40$ ,  $50$ , and  $60^{\circ}\text{C}$ ) for 5 days. Each drying temperature treatment consisted of 4–5 repetitions.

### Extraction and determination of the four medicinal compounds

The dried root blocks from all treatments and controls were powdered for extraction. According to the extract method described by Li et al. (2008) with some modifications, the root powder was extracted with 60% (v/v) ethanol for 30 min in an ultrasonic bath, and the ratio of solid to liquid was 1 g DW/100 ml. The recoveries of GL, LQ, LG, and ISLG were 94%, 94%, 96%, and 97%, respectively. The solid matter was removed by centrifugation at 15,000 rpm for 10 min. Then, the supernatant was filtered through a  $0.2\text{ }\mu\text{m}$  syringe filter. Finally, the filtered supernatant was injected into the high-performance liquid chromatography (HPLC) column, and concentrations of the four compounds were calculated from the peak areas.

The HPLC quantification method for GL, LQ, LG, and ISLG described by Li et al. (2008) was modified and was used for the present experiment. In brief, quantification was performed on a Shimadzu 10AD HPLC system equipped with an SCL-10A system controller, SIL-10A auto-injector, and CTO-10A column oven with ultraviolet (UV) detection at 254 nm for GL, 276 nm for LQ and LG, and 372 nm for ISLG in an SPD-M10AV photodiode array detector (Shimadzu Co., Ltd., Japan). The four compounds were separated on a TSK-Gel ODS-100V reversed-phase column (4.6 mm diameter  $\times$  250 mm length) (Shimadzu Co., Ltd., Japan). The mobile phase consisted of acetonitrile and 1% acetic acid. Baseline separation of GL, LQ, LG, and ISLG was achieved with gradient elution. Flow rate was 0.9 ml/min, injection volume was 20  $\mu\text{l}$ , and column temperature was maintained at  $40^{\circ}\text{C}$ . Standards of GL (PMRJ, Japan) and LQ, LG, and ISLG (ChromaDex, Inc., USA) were dissolved in methanol to obtain different solution concentrations. Chromatographic peaks of the four compounds were confirmed by comparing their retention times and UV spectra with those of the standards. Standard curves based on the standards showed good linearity over ranges of 37.5–600.0  $\mu\text{g/ml}$  for GL, 15.6–250.0

$\mu\text{g/ml}$  for LQ, 0.3–10.0  $\mu\text{g/ml}$  for LG, and 0.05–1.56  $\mu\text{g/ml}$  for ISLG. Amounts of the four compounds in every unknown sample were determined using standard curves.

#### Calculation

To elucidate concentration trends in the four medicinal compounds between the storage treatment or drying temperature and the controls, the increment percentage in the compounds with respect to controls ( $P_{\text{inc}}$ , %) was calculated according to the following equation:

$$P_{\text{inc}} = (C_T - C_c) / C_c \times 100$$

$P_{\text{inc}}$ : the increment percentage in the compound concentration with respect to control (%).

$C_T$ : the compound concentration in each storage or drying temperature treatment (mg/g DW).

$C_c$ : the compound concentration in each control (mg/g DW).

#### Statistical analysis

All data presented in the figures are mean values. Significant differences between temperature treatments (storage and drying temperatures) and controls for the same periods were determined by t-test ( $P \leq 0.01$  or 0.05) using Excel Statistics ver. 5.0 software (ESUMI Co., Ltd., Japan).

## RESULTS AND DISCUSSION

The four medicinal compounds were completely separated from impurities under HPLC conditions (Fig. 2). Reported concentration ranges of GL, LQ, LG, and ISLG in wild Chinese licorice are 14.8–33.4, 4.1–26.6, 0.7–3.5, and 0.3–1.2 mg/g DW, respectively (Chen et al., 2009; Zhao et al., 2006). GL and LQ concentrations in our study were consistent with reported concentrations in wild Chinese licorice, but LG and ISLG concentrations were one-tenth of those reported. In our previous studies, the four compound concentrations were almost the same between hydroponic and soil cultivations. Although the reason for the low LG and ISLG concentrations was not clear, the composition of the four compounds in the line (No. 2) used in our study may have been different from that used in the above re-

ports.

#### Effects of post-harvest storage temperature on concentrations of the four medicinal compounds

Generally, most enzyme activities decrease by decreasing the amount of liquid in plants stored below the freezing point (about 0°C). Therefore, the synthesis or decomposition of enzyme activity for the four medicinal compounds in this experiment may change under below- or above-zero temperature conditions via liquid movement. The results of the below-zero (−80, −30, and −13°C) and above-zero (4 and 25°C) treatments in this experiment are discussed in the following sections.

#### Below-zero treatments

Concentrations and increment percentages ( $P_{\text{inc}}$ ) of the four medicinal compounds in roots stored at −80°C for 1–4 weeks were not changed significantly compared to those in the controls, except for LQ at 3 weeks and ISLG at 2 weeks (Fig. 3). Little liquid seemed to exist in the roots of Chinese licorice stored at −80°C. Therefore, plant biosynthesis and most enzyme reactions also seemed to be stopped at −80°C.

Hayashi (2010) reported that GL concentrations in harvested Chinese licorice roots stored in the incubator at −5°C for 4 weeks and in the storehouse at ambient temperatures in winter (−15 to 8°C) for 8 weeks were 7% and 12% higher, respectively, than GL concentrations in roots dried directly. In our study, GL and LQ concentrations in roots stored at −30 and −13°C for 1–2 weeks tended to be 4–13% higher than GL and LQ concentrations in roots dried directly in the vacuum freeze dryer (controls) (Fig. 3A and 3B). The  $P_{\text{inc}}$  of GL and LQ concentrations fluctuated in roots stored at −30 and −13°C for 1–4 weeks. For Chinese licorice, GL and LQ were the two secondary metabolites in the high-concentration group. Generally, secondary metabolites have a key role in protecting plants from environmental pressures or controlling plant growth (Harborne, 1999). When roots were stored at −30°C, additional GL and LQ were produced to resist the low temperature and then were consumed gradually. GL and LQ concentrations in roots stored at −30°C increased in the

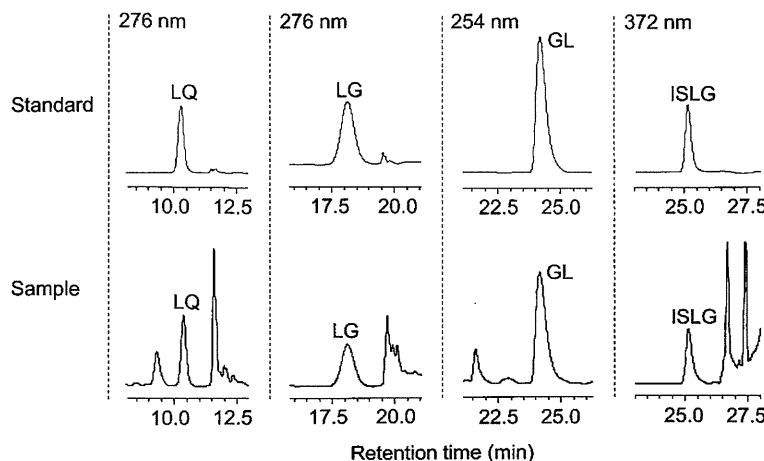
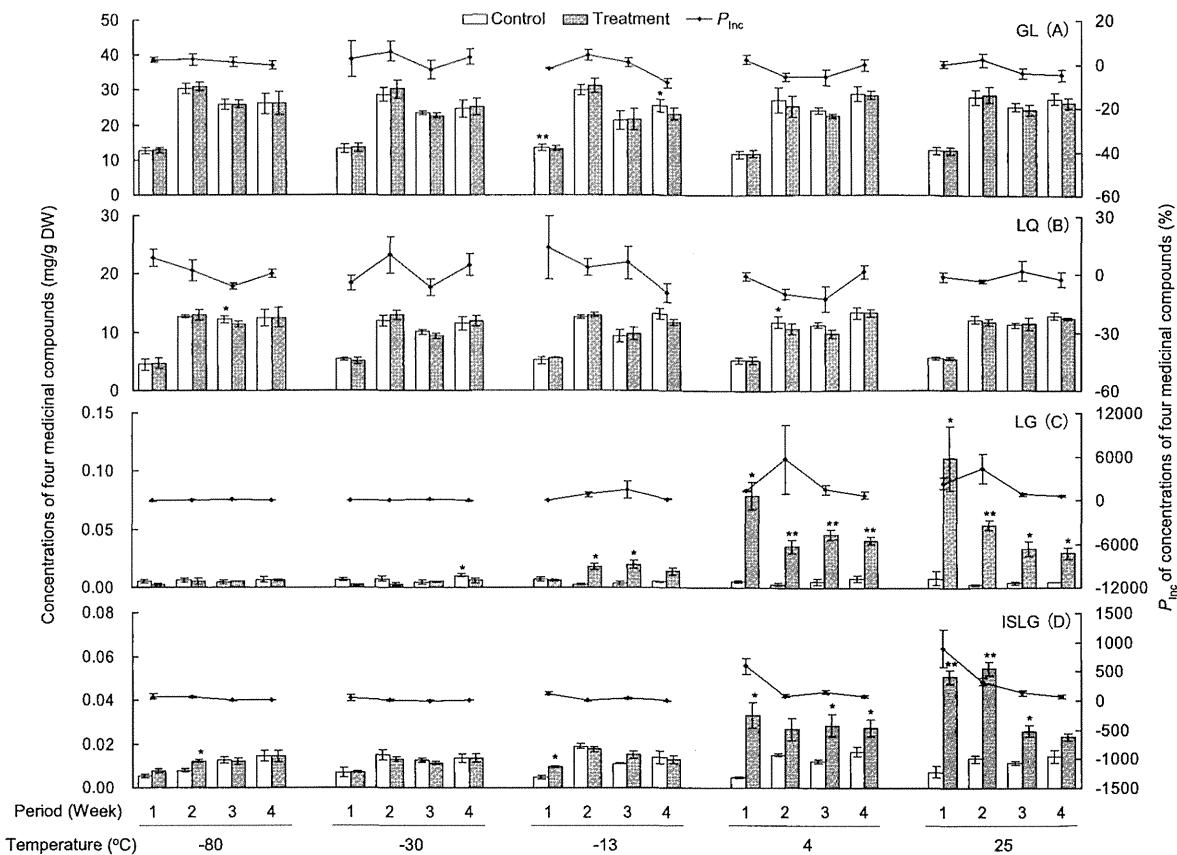


Fig. 2 HPLC chromatograms of four standards and one sample. Peaks: GL, glycyrrhetic acid; LQ, liquiritin; LG, liquiritigenin; ISLG, isoliquiritigenin. Mobile phase: acetonitrile and 1% acetic acid. Flow rate: 0.9 ml/min. UV detection: 254 nm for GL, 276 nm for LQ and LG, and 372 nm for ISLG. Column temperature: 40°C.



**Fig. 3** Effects of post-harvest root storage temperatures ( $-80$ ,  $-30$ ,  $-25$ ,  $-13$ ,  $4$ , and  $25^{\circ}\text{C}$ ) on concentrations and increment percentages ( $P_{\text{inc}}$ ,  $\rightarrow$ ) of the four medicinal compounds in dry roots of Chinese licorice for 1–4 weeks after the beginning of temperature treatment. One part of the root as controls (control,  $\square$ ) was dried directly in a vacuum freeze dryer, and the other part (treatment,  $\blacksquare$ ) was stored at different temperatures for 1, 2, 3, and 4 weeks and then dried in a vacuum freeze dryer. A: glycyrrhetic acid (GL); B: liquiritin (LQ); C: liquiritigenin (LG); D: isoliquiritigenin (ISLG). \* and \*\* indicate significant differences between treatments and controls for each treatment at  $P \leq 0.05$  and  $P \leq 0.01$  by t-test. Each bar indicates mean  $\pm$  S.E. ( $n=3$ –4).

second week and decreased in the third week (Fig. 3A and 3B). Then, the plants continued to produce more GL and LQ to resist the low temperature ( $-30^{\circ}\text{C}$ ). GL and LQ concentrations began to increase again in the fourth week. However, when roots were stored at  $-13^{\circ}\text{C}$ , additional GL was produced in the second and third weeks and was consumed in the fourth week, and additional LQ was produced in the first to third weeks and was consumed in the fourth week (Fig. 3A and 3B). Probably because  $-13^{\circ}\text{C}$  is warmer than  $-30^{\circ}\text{C}$ , consumption rates of GL and LQ in roots stored at  $-13^{\circ}\text{C}$  were slower than those in roots stored at  $-30^{\circ}\text{C}$ . These results suggest that fluctuations in GL and LQ concentrations in Chinese licorice stored at  $-30$  and  $-13^{\circ}\text{C}$  were related to plant protection.

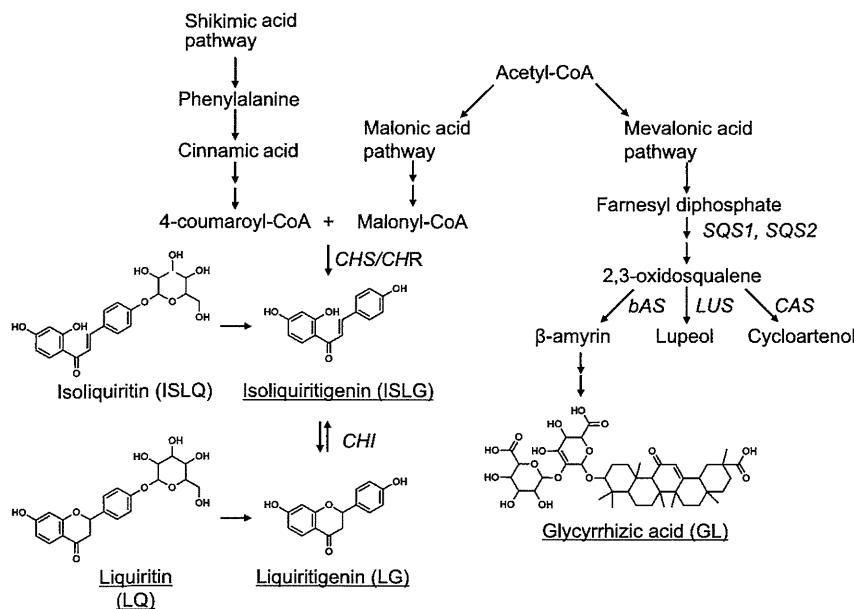
LG and ISLG concentrations in roots stored at  $-30^{\circ}\text{C}$  for 1–4 weeks were not significantly changed compared to those in the controls, except for LG at 4 weeks (Fig. 3C and 3D). The  $P_{\text{inc}}$  of LG and ISLG concentrations tended to be higher in roots stored at  $-13^{\circ}\text{C}$  than in roots stored at  $-30^{\circ}\text{C}$  (Fig. 3C and 3D). However, the  $P_{\text{inc}}$  of LG and ISLG concentrations also fluctuated like the  $P_{\text{inc}}$  of GL and LQ concentrations, although LG and ISLG were the two secondary metabolites in the low-concentration group.

#### Above-zero treatments

LG and ISLG concentrations in roots stored at 4 and

$25^{\circ}\text{C}$  were significantly ( $P \leq 0.01$  and  $0.05$ ) higher than those in the controls, except for ISLG concentration in roots stored at  $4^{\circ}\text{C}$  for 2 weeks and at  $25^{\circ}\text{C}$  for 4 weeks (Fig. 3C and 3D). The  $P_{\text{inc}}$  of LG concentration was highest in roots stored at 4 and  $25^{\circ}\text{C}$  for 2 weeks, and LG concentration was nearly 60-fold (6000%) higher than that in the controls (Fig. 3C). The  $P_{\text{inc}}$  of ISLG concentration was higher in roots stored at 4 and  $25^{\circ}\text{C}$  for 1 week than in roots stored for 2–4 weeks, and ISLG concentration was nearly 10-fold (1000%) higher than that in the controls (Fig. 3D). However, LQ concentration decreased when roots were stored at 4 and  $25^{\circ}\text{C}$  (Fig. 3B). LQ, LG, and ISLG are flavonoids. Generally, certain flavonoids occur in combination with glucoses as glucosides, and free flavonoids are released when exposed to certain enzymes or stimulation (Fu et al., 2008). The flavonoids LQ and isoliquiritin (ISLQ) in Chinese licorice as glucosides possibly could have decomposed to free flavonoids (Fig. 4). LG and ISLG, when roots were stored at 4 or  $25^{\circ}\text{C}$ . This would explain the decrease in LQ concentration and the increase in LG and ISLG concentrations in roots stored at 4 and  $25^{\circ}\text{C}$  for 1–4 weeks.

GL is a triterpenoid. GL and the other three flavonoids (LQ, LG, and ISLG) are synthesized in Chinese licorice through the action of certain enzymes by different



**Fig. 4** Biosynthetic pathway of the four medicinal compounds (Hayashi et al., 2003; Taiz and Zeiger, 2010; Winkel-Shirley, 2001). The underlined compounds were the target compounds in this study. *CHS*: chalcone synthase; *CHR*: chalcone reductase; *CHI*: chalcone isomerase; *SQS*: squalene synthase; *bAS*:  $\beta$ -amyrin synthase; *LUS*: lupeol synthase; *CAS*: cycloartenol synthase.

biosynthetic pathways (Fig. 4) (Winkel-Shirley, 2001; Hayashi et al., 2003; Taiz and Zeiger, 2010). Secondary metabolites are known to be derived from primary metabolites through the activity of enzymes. Acetyl coenzyme A (acetyl-CoA) is the most important enzyme for the synthesis of secondary metabolites in plants. Therefore, a possible reason for the decreased GL concentration in roots stored at 4 and 25°C was that more acetyl-CoA in the plant was used to synthesize the flavonoids (LG and ISLG) instead of the triterpenoid (GL). However, plant biosyntheses are complex, and this mechanism needs to be studied further.

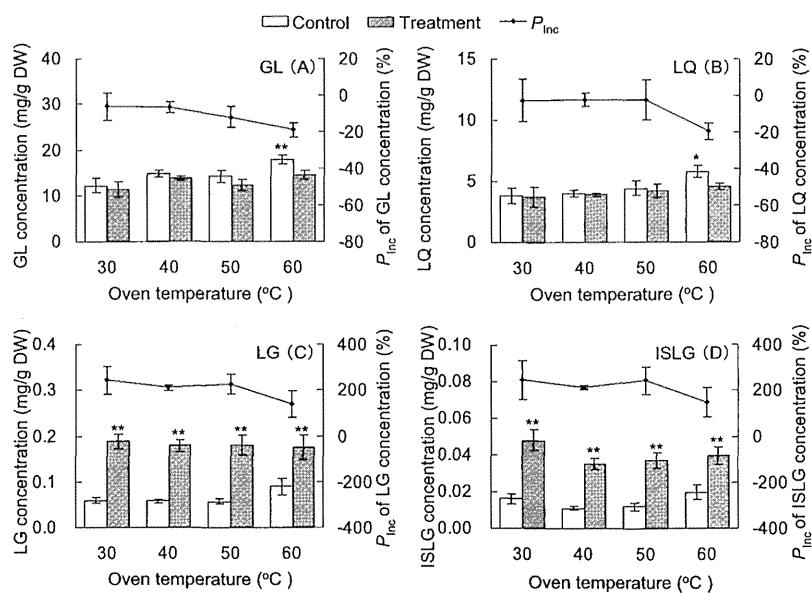
#### All treatment ranges

Most of the enzyme reactions in the roots of Chinese licorice stored at  $-80^{\circ}\text{C}$  were stopped without liquid movement; therefore, the  $P_{\text{inc}}$  of the four medicinal compounds in roots stored for 1–4 weeks was stable (Fig. 3). At  $-30$  and  $-13^{\circ}\text{C}$ , some enzyme activity seemed likely; therefore, the  $P_{\text{inc}}$  of GL and LQ concentrations in roots stored at  $-30$  and  $-13^{\circ}\text{C}$  fluctuated, and the  $P_{\text{inc}}$  of LG and ISLG concentrations in roots stored at  $-13^{\circ}\text{C}$  tended to increase. At above-zero temperatures, because most of the enzyme still maintained activity, the  $P_{\text{inc}}$  of GL and LQ concentrations tended to decrease (Fig. 3A and 3B) and the  $P_{\text{inc}}$  of LG and ISLG concentrations increased (Fig. 3C and 3D). According to these results, the concentrations of medicinal compounds in roots stored at different temperatures changed. Roots with stable concentrations of the four medicinal compounds were obtained at  $-80^{\circ}\text{C}$ , and roots with high concentrations of the four medicinal compounds were obtained at  $-30^{\circ}\text{C}$  for 2 weeks for GL,  $-13^{\circ}\text{C}$  for 1 week for LQ,  $4^{\circ}\text{C}$  for 2 weeks for LG, and  $25^{\circ}\text{C}$  for 1 week for ISLG. In our previous studies in which we used different-aged seedlings for different periods, the results were consistent with the above results in this study.

#### Effects of drying temperature on concentrations of the four medicinal compounds

Compound concentrations in the medicinal parts of plants dried in a vacuum freeze dryer can be steadily maintained; however, oven drying is a popular method for the medicinal plant production industry because of short drying time and ease of temperature control. Drying temperature affects the quality and concentration of active compounds in medicinal plants. In commercial operations in Canada, ginseng roots are dried at low temperatures ( $32$ – $38^{\circ}\text{C}$ ) to achieve a high-quality product (Davidson et al., 2004). Lin et al. (2011) reported that hot drying temperatures ( $40$ ,  $55$ , or  $70^{\circ}\text{C}$ ) decreased caffeic acid derivatives and total phenolics contents, and a cool drying temperature ( $30^{\circ}\text{C}$ ) tended to increase eichoric acid and total phenolics contents in the roots of *Echinacea purpurea*.

In our study, drying temperature also affected the concentrations of medicinal compounds in the roots of Chinese licorice. GL and LQ concentrations in roots dried in the oven at  $60^{\circ}\text{C}$  were significantly ( $P \leq 0.01$  and  $0.05$ ) lower than those in roots dried in the vacuum freeze dryer (controls) (Fig. 5A and 5B), and the  $P_{\text{inc}}$  of GL concentration in roots dried at  $50$  and  $60^{\circ}\text{C}$  tended to decrease (Fig. 5A). These results indicate that GL and LQ in roots dried at high temperatures ( $50$  and  $60^{\circ}\text{C}$ ) decomposed to other related compounds. However, because LG and ISLG concentrations in roots dried in the oven at  $30$ – $60^{\circ}\text{C}$  were significantly ( $P \leq 0.01$ ) higher than those in the controls (Fig. 5C and 5D), LQ and ISLG in roots dried at  $30$ – $60^{\circ}\text{C}$  probably decomposed to LG and ISLG (Fig. 4). In addition, the  $P_{\text{inc}}$  of LG and ISLG concentrations tended to be lower in roots dried at  $60^{\circ}\text{C}$  than in roots dried at  $30$ – $50^{\circ}\text{C}$  (Fig. 5C and 5D), which indicates that LG and ISLG also decomposed when roots were dried at temperatures above  $60^{\circ}\text{C}$ . These results suggest that drying Chinese licorice root in



**Fig. 5** Effects of post-harvest drying temperatures (30, 40, 50, and 60°C) on concentrations and increment percentages ( $P_{inc}$ ,  $\rightarrow$ ) of the four medicinal compounds in dry roots of Chinese licorice. One part of the root as control (control,  $\square$ ) was freeze-dried, and the other part (treatment,  $\blacksquare$ ) was oven dried at different temperatures. A: glycyrrhetic acid (GL); B: liquiritin (LQ); C: liquiritigenin (LG); D: isoliquiritigenin (ISLG). \* and \*\* indicate significant differences between the two drying methods at  $P \leq 0.05$  and  $P \leq 0.01$  by t-test. Each bar indicates mean  $\pm$  S.E. ( $n=4-5$ ).

the oven at 30 and 40°C with low energy consumption could increase LG and ISLG concentrations without decreasing GL and LQ concentrations. In our previous studies in which roots were dried in the oven at 50°C, the results were consistent with the above results in this study, although other drying temperature treatments (30, 40, and 60°C) were not repeated.

## CONCLUSION

In this study, concentrations and compositions of GL, LQ, LG, and ISLG were changed using different storage temperature treatments. Concentrations of the four medicinal compounds were stable at  $-80^{\circ}\text{C}$ . GL and LQ concentrations in the root of Chinese licorice can be increased by below-zero storage temperature treatments ( $-30$  and  $-13^{\circ}\text{C}$ ), and LG and ISLG concentrations can be increased by above-zero storage temperature treatments (4 and  $25^{\circ}\text{C}$ ). In addition, after above-zero storage temperature treatments, LG and ISLG concentrations can be increased more by low-temperature drying (30–40°C). This study provides an approach to increase the concentration of each target compound in Chinese licorice for different market demands (drugs, cosmetics, and food).

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特集

日常生活とアレルギー

コチニール色素とアレルギー

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## コチニール色素とアレルギー

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### はじめに

平成24(2012)年5月11日、消費者庁から「コチニール色素に関する注意喚起」として、コチニール色素が添加された食品を摂取したとき、急性アレルギー反応(アナフィラキシー)を引き起こした症例の研究情報の提供があったと報告された。アナフィラキシーを発症した場合、呼吸困難などの重篤な症状となる場合もあるため注意が必要となる。コチニール色素は、赤色の着色を目的として、食品添加物だけでなく医薬部外品や化粧品などさまざまな用途で使用されている。消費者庁の注意喚起を受けて、何らかのアレルギー症状の既往歴のある消費者は、コチニール色素が含まれているか否かの表示を確認することが必要と考えられる。また消費者にコチニール色素の種類や用途範囲の正確な情報を伝えることが、リスク管理上重要であると考えられた。

本稿では、レギュラトリーサイエンスの観点から、わが国と諸外国の規格を基に、コチニール色素とそのアルミニウム結合物(レーキ)であるカルミンがどのような色素であるかについて説明する。また、最近の知見を交えながら、コチニール色素とアレルギーの関係について解説する。

### コチニール色素とは

コチニール色素は、中南米の砂漠地帯を主要産

地とし、その他に西インド、カナリア諸島、スペインなどの地域でも産するが、ペルー1国で世界の90%以上が産出される。南米ではインカ帝国の時代から衣服や装飾を赤色に染める目的で使用されてきたもので、サボテン(学名: *Nepalea coccinellifera*)に生息する雌のエンジムシ(別名: コチニールカイガラムシ)【学名: *Dactylopius coccus* Costa (*Coccus cacti* Linnaeus)】を収穫期に刷毛で落として収穫する。熱湯で殺した虫を天日で乾かしたものが黒色コチニールで、貯蔵している間にコチニール・グランコ(銀色コチニール)に変わるとされている<sup>1)</sup>。この乾燥体より、温時~熱時水で、または温時含水エタノールで抽出して得られたものがコチニール色素である。コチニール色素の赤色の主色素成分はカルミン酸(carmine acid)であり、耐熱性、耐光性に優れていることから、天然由来の赤色の着色料として古くから使用されている。カルミン酸の化学構造を図1に示したが、アントラキノン骨格を有する化合物であり、コチニール色素にはこのカルミン酸が数%程度含まれるとされている。

また、コチニール色素の主色素成分であるカルミン酸にアルミニウムなどを加えて不溶化(レーキ化)したもの、すなわち、カルミン酸のアルミニウムレーキやアルミニウム・カルシウムレーキをカルミン(carmine)という。JECFA [FAO/WHO 合同食品添加物専門家委員会(Joint FAO/

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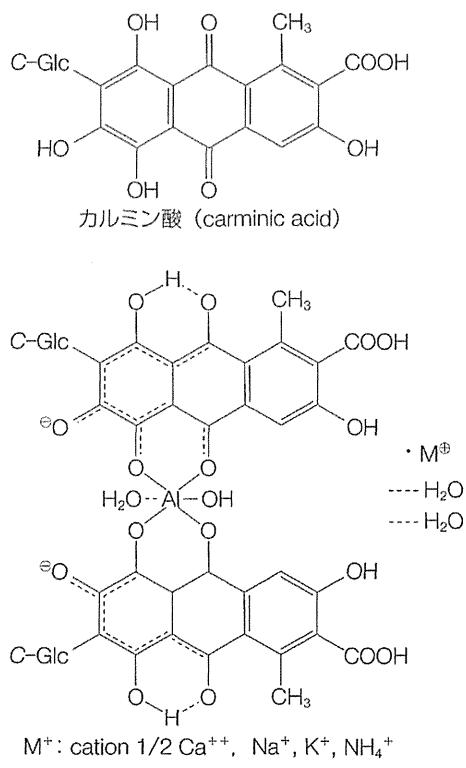


図1 カルミン酸とカルミンの構造

WHO Expert Committee on Food Additives)】および欧州連合(EU)の成分規格には、図1に示すカルミンの推定化学構造が示され、アルミニウムとカルミン酸の推定比率1:2の水酸化アルミニウムレーキとされており、このほかにカルシウム、ナトリウム、カリウム、アンモニウムなどの複合塩として存在するとされている<sup>2)</sup>。カルミンには約20~50%のカルミン酸が含まれるとされている。

コチニール色素、カルミンとともに、一般毒性試験および特殊毒性試験の安全性に関するデータも多く報告され、高い安全性が知られていることから、諸外国で広く使用されている着色料の1つである。JECFAは、カルミンの1日摂取許容量を体重1kg当たり5mgと評価している。

### コチニール色素の成分規格とその用途

わが国では、化学合成による色素(タール色素)

の安全性が過去に問題視されたこともあり、国民の天然志向は強く、天然由来の色素としてpHによって多彩な赤色を示すコチニール色素は合成赤色素の代わりに用いられることが多い。ただし、コチニール色素とカルミンについてはわが国の規制上、その用途は大きく異なる。

コチニール色素は食品衛生法の既存添加物名簿に収載されており、食品添加物として使用すること認められている。わが国で使用できる食品添加物の成分規格や基準は「食品、添加物等の規格基準」(厚生省告示370号)で定められており、食品添加物公定書(現時点では第8版が刊行されている)にまとめられている<sup>3)</sup>。わが国では天然香料および一般飲食物添加物以外の天然由来の食品添加物(化学的合成品以外)を、行政上、既存添加物と呼ぶ。既存添加物とは、1995年の食品衛生法及び栄養改善法の一部を改正する法律(平成7年法律第101号)附則第2条第4項で規定される既存添加物名簿に収録された食品添加物のことを指し、化学的手段により元素または化合物に分解反応以外の化学反応を起こさせて得られた物質以外のものとされている。よって、カルミンはコチニール色素を化学反応によりレーキ化させたものに当たるため、天然物とみなされず、わが国では、現時点では食品添加物としての使用は許可されていない。カルミンはコチニール色素とともに医薬部外品原料規格に収載されており、医薬部外品、化粧品には使用できることとなっている<sup>4)</sup>。よって、法規制上、わが国ではコチニール色素は食品だけでなく医薬部外品や化粧品などに使用できるが、カルミンは食品に用いてはならないということになっている。表1にコチニール色素とカルミンの用途、使用対象、成分表示および使用基準を示した。

これに対して、多くの諸外国では法規制が異なり、コチニール色素およびカルミンはともに食品や医薬部外品、化粧品などへの使用が許可されている。実際、欧米諸国などにおいて、カルミンはハムやソーセージなどの食品をはじめ医薬品、医薬部外品、化粧品などの着色料として広く使用さ