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Adulteration Identification of Citrus Juice by Denaturing Gradient Gel Electrophoresis (DGGE)

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ABSTRACT

*trn*L intron and *trn*L-*trn*F non-coding region have become powerful tools to identify plants species in the past few years. In this study, above two regions are used to identify oranges and mandarins via a variable DNA fragment in *trn*L intron. In order to identify the DNA, a primer pair was designed to amplify the variable fragments and the PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE). The method successfully detected other citrus juices mixed in all of the falsely claimed 100% orange juices. It is suggested that the method is useful to detect the adulteration of 100% pure orange juices.

Key words: *trn*L, DGGE, citrus, chloroplast

INTRODUCTION

Orange juice is the most popular juice worldwide. According to the statistics of the Food and Agriculture Organization (FAO), the export of concentrated citrus juice in 2002 was US$ 6.2 billion and the output of citrus juice increased by 50% from 2000 to 2002. Adulteration of juices is a recurrent problem, which has received extensive media attention. Furthermore, recent health problems related to food industry have also increased consumer concerns[(1)]. Common methods of adulteration include addition of water, less expensive juices, pulp wash, colorants, and other undeclared additives either alone or in combination to replicate the composition profiles of pure juices[(2,3)]. Numerous methods of detecting juice adulteration have been developed, such as HPLC[(4,5)], capillary electrophoresis[(1)] and pyrolysis mass spectroscopy[(6)], that analyze the chemical components of juice. Analyzing a single natural juice component is inadequate in obtaining sufficient information to determine juice purity; therefore, multiple component chemical analyses are required to accurately evaluate reliably the differences between adulterated and pure juices. This approach, however, is both time-consuming and expensive[(6)].

Currently, DNA markers are extensively employed for taxonomy study in many plants[(7,8)]. These DNA markers provide evidences for plant species identification and are useful for analyzing commercial fruit products. Several DNA markers have been used in citrus analysis, such as Random Amplified Polymorphic DNA (RAPD)[(9,10,11)], Restriction Fragment Length Polymorphism (RFLP)[(12,13)], Sequence-Characterized Amplified Regions (SCARs), and chloroplast DNA (cpDNA)[(14)]. Among these, cpDNA analysis is especially effective in phylogenetic analysis due to its evolutionary conservatism, relative abundance in plant tissue, small size and predominant uniparental inheritance[(15)]. The most common chloroplast gene utilized to obtain sequence data for cladistic analyses in plants is the large subunit of the ribulose-1,5-bisphosphate carboxylase / oxygenase gene (*rbc*L)[(16)]. However, when employed alone, *rbc*L is less suitable at lower taxonomic levels than more rapidly evolving genes, introns, and spacers, such as the non-coding region of the chloroplasts DNA *trn*L (leucine) intron and *trn*L-*trn*F (phenylalanine) intergenic spacer[(17,18,19)].

Denaturing gradient gel electrophoresis (DGGE) separates PCR amplicons of similar length with dissimilar nucleotide compositions on a denaturing gradient gel[(20)]. The DGGE system can be divided into the perpendicular and parallel DGGE, which are differentiated by the direction of denaturant gradient and electrophoresis. In this study, *trn*L intron and *trn*L-*trn*F intergenic spacer are amplified with a specific primer and the PCR products are employed to run the DGGE. This method is useful and accurate, and can be used in fruit industry.

MATERIALS AND METHODS

I. *Plant Materials and DNA Extraction*

Ten genotypes of *Citrus*, six oranges (*Citrus sinensis* (L.) Osbeck) and four mandarins (*Citrus reticulata* Blanco) are used in this study. More specifically six oranges are Liucheng, Pineapple, Valencia, Hamlin, Parson and Navel Seeding and four mandarins are Ponkan, Tankan, Satsuma Seeding and Murcoot. These plant materials were provided by the National Plant Genetic Resource Center of Taiwan. For sequencing, total DNA was extracted from young leaves according to the method described in Doyle and Doyle[(21)].

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For DGGE, DNA was extracted from citrus juice according to the method developed by Lipp *et al.*[22].

II. *Amplification and Sequencing of trnL Intron in Chloroplast DNA*

The cpDNA of the 10 genotypes were amplified using one pair of universal primers[23]. The PCR conditions for a total volume of 25 μL were as follows: 200 ng of template DNA; 10× buffer; 6.25 pmoles/each; 0.1 mM dNTP; and 0.5 Unit DynaZyme. The parameters of the amplification reaction were: 1 cycle of 3 min at 96°C; 30 cycles of 1 sec at 96°C, 10 sec at 54°C, 20 sec at 72°C, and an end cycle of 10 min at 72°C. Sequences were generated on an ABI automated sequencer from the Mission Biotech Co. Ltd. (Taiwan) employing the same primers as in amplification. The sequences were aligned with a BioEdit Sequence Alignment Editor.

III. *Oligonucleotide Primers and PCR Condition*

Synthesized primers supplied by the Mission Biotech Co. Ltd. (Taiwan) were diluted with an appropriate volume of water to a final concentration of 100 μmol/L and stored at -20°C until use. The primer pair (B49317 and A50272) designed by Pierre *et al.*[23] was used to amplify the cpDNA

(A)

Mandarin CGAAATCGGTAGACGCTACGGACTTAATTGGATTGAGCCTTAGTATGGAA-CTTTCTAAG 59
orange CGAAATCGGTAGACGCTACGGACTTAATTGGATTGAGCCTTAGATTGGAAACTTACTAAG 60

Mandarin TGATAACTTTCAAATTCAGAGAAACCCAGGAATTAAAAATGGGTAATCCTGAGCCAAATC 119
orange TGATAACTTTCAAATTCAGAGAAACCCAGGAATTAAAAATGGGTAATCCTGAGCCAAATC 120

Mandarin CTCTTCTCTTTTCCAAGAACAAACAGGGGTTCAGAAAGCGAAAAAGGGGGATAGGTGCAG 179
orange CTCTTCTCTTTTCCAAGAACAAACAGGGGTTCAGAAAGCGAAAAAGGGGGATAGGTGCAG 180

Mandarin AGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGACTGCCCTTTTTGGTAAAGAAAAAA 239
orange AGACTCAATGGAAGCTGTTCTAACAAATGGAGTTGACTGCCCTTTTTGGTAAAGAAAAAA 240

Mandarin GAAA**GTAAAATGAATGCTTCTATCG**AATATCGAAACTCGATAAAGGATGAAGGATAAGGG 299
orange GAAA**GTCAAATGAATGCTTCTATCG**AATATCGAAACTCGATAAAGGATGAAGGATAAGGG 300

Mandarin TATATAGACTATGGATACGCAGCGAAAAACTAACTCAAAAATCACAACCAAATACGTATT 359
orange TATATAGACTATGTATACGCAGCGAAAAACTAACTCAAAAATCACAACCAAATACGTATT 360

Mandarin CCTTTTTATGAAAAAGAAAAAGAAAAGAATTGTTATTGTTATGAATCGATTCTAAGTTGA 419
orange TCTTTTTATGAAAAAGAAAA------GAATTGGTATTGTTATGAATCGATTCTAAGTTGA 414

Mandarin AGAAAGAATCGAATATTCCCGGCTCAAATCATTCACTCCACCTCCATGGTCTGATCGATC 479
orange AGAAAGAATCGAATATTCCCGGCTCAAATCATTCACTCCACCTCCATGGTCTGATCGATC 474

Mandarin CTTTCTTTTGACTTTTGAAGAACTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTC 539
orange CTTTCTTTTGACTTTTGAAGAACTGATTAATCGGACGAGAATAAAGATAGAGTCCCATTC 534

Mandarin TACATGTCAATATCAATACGGGCAACAATGAAATTTAGAGTAAAAGGAAAATCCGTCGAC 599
orange TACATGTCAATATCAATACGGGCAACAATGAAATTTAGAGTAAAAGGAAAATCCGTCGAC 594

Mandarin TTTAGAAATC**GTGAGGGTTCAAGTCCCT**CTATCCCCA 636
orange TTTAGAAATC**GTGAGGGTTCAAGTCCCT**CTATCCCCA 631

(B)

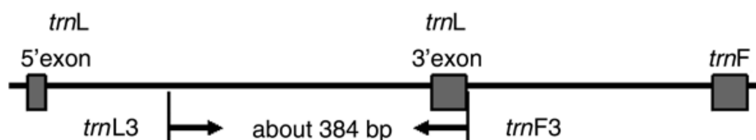

Figure 1. (A) The *trn*L intron sequence of orange and mandarin. The boldface is the primer sequence. (B) Position of *trn*L3/*trn*F3 primer in *trn*L intron.

Table 1. The universal primers and specific primers used for amplifying the non-coding regions of chloroplast DNA

Primer	Sequence 5'-3'	PCR product	Primer source reference
B49317	CGAAATCGGTAGACGCTACG	About 1070 bp	Pirre *et al.*[23]
A50272	ATTTGAACTGGTGACACGAG		Pirre *et al.*[23]
*trn*L3	GTCAAATGAATGCTTCTATCG	About 384 bp	This study
*trn*F3	AGGGACTTGAACCCTCAC		This study

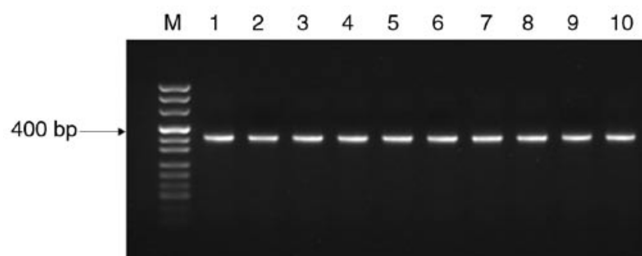

Figure 2. Amplification of the variable *trn*L intron region in six oranges and four mandarins by *trn*L3/*trn*F3 primer. Lane M: 50 bp ladder markers; Lane 1: Liucheng; Lane 2: Pineapple; Lane 3: Valencia; Lane 4: Hamlin; Lane 5: Parson; Lane 6: Navel Seeding; Lane 7: Ponkan; Lane 8: Tankan; Lane 9: Satsuma Seeding; Lane 10: Murcoot.

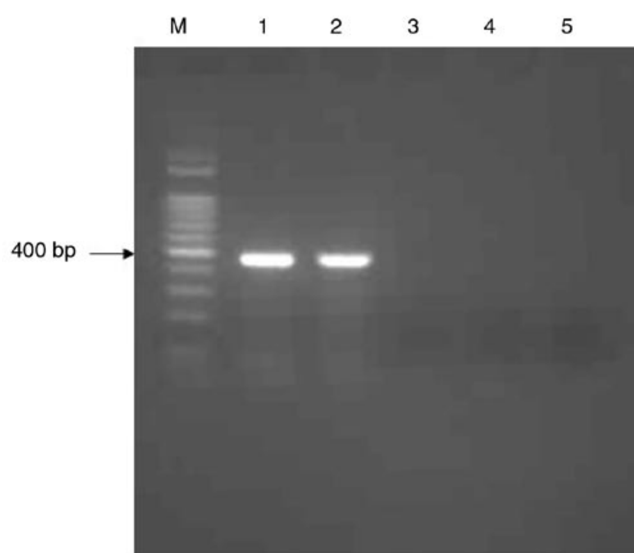

Figure 3. The specificity test of *trn*L3/*trn*F3 primer. Lane M: 100 bp ladder marker; Lane 1: Liucheng (orange); Lane 2: Tankan (mandarin); Lane 3: maize; Lane 4: sugar cane; Lane5: no temple control.

for sequencing. The second primer pair *trn*L3/*trn*F3 was designed and synthesized to amplify the differentiating fragments in oranges and mandarins. The parameters of the amplification reaction were: 1 cycle of 3 min at 96°C; 35 cycles of 1 sec at 96°C, 10 sec at 52°C, 20 sec at 72°C, and an end cycle of 10 min at 72°C. The primer pair *trn*L3/*trn*F3 was designed for DGGE.

IV. *Denaturing Gradient Gel Electrophoresis*

Denaturing gradient gel electrophoresis was performed as described previously[20]. Briefly, 10% polyacrylamide gel (25 mL) with a linear gradient of 15~35% denaturant were poured between glass plates separated by teflon spacers (0.75 mm thick), with a sample well width of 8 mm. Gel was run in DCode^{TM} Universal Mutation Detection System (Bio-Rad, USA) and immersed in an aquarium of running buffer (20 mM sodium acetate, 1 mM EDTA, 40 mM Tris-acetate, pH 7.4) that was maintained at 60°C with a circulating heater. Following electrophoresis for 3 hr at 150 V, the gel was stained with ethidium bromide and photographed.

RESULTS AND DISCUSSION

I. *Primer Design*

The non-coding regions of chloroplast have recently been employed to study the population biology and evolution of plants, and most studies have suggested that these regions could be useful markers for species identification of plants. Araujo *et al.*[24], who utilized three non-coding regions of chloroplast DNA, *trn*T-*trn*L intergenic spacer, *trn*L intron and *trn*L-*trn*F intergenic spacer, to identify the phylogenesis of Citreae, showed that the oranges (*Citrus sinensis*) and mandarins (*Citrus reticulata*) could be divided into two groups. In this study, the chloroplast DNA *trn*L intron and *trn*L-*trn*F intergenic spacers of six orange species and four mandarin species were amplified with the universal primers B49317 and A50272 (Table 1) and the length of the PCR products were roughly 1070 bp (data not shown). These DNA fragments, which were applied for sequencing and were analyzed by GeneDoc, showed a variable DNA fragment between oranges and mandarins (Figures 1A and 1B). Based on the conserved sites flanking the variable regions within *trn*L intron, the primer pair *trn*L3/*trn*F3 (Table 1) was further designed to amplify a 384 bp fragment from all our orange and mandarin samples. It was found that the primer pair could be applied to all samples (Figure 2). To verify the species-specificity of this primer pair, the citrus, maize and sugar cane were tested by PCR and there was no PCR products detected other than citrus (Figure 3). The maize and the sugar cane were used here to confirm that their respective DNA does not interfere with the findings of the study. Experimental results showed that the primer pair *trn*L3/*trn*F3 is specific to citrus, and it can amplify the variable region of *trn*L intron in all citrus samples.

II. *Condition of Denaturing Gradient Gel Electrophoresis*

The PCR products amplified by the *trn*L3/*trn*F3 primer were used to run the perpendicular DGGE. The goal of running the perpendicular DGGE is to optimize electrophoresis condition. Figure 4 shows the perpendicular DGGE with a denaturing gradient ranging from 0 to 100%. The optimal range for separating the oranges and mandarins was between 15 and 35%. This range was applied to the parallel DGGE for identifying the oranges and mandarins. The DNA fragments of oranges and mandarins were located at different sites on the parallel denaturing gradient gel according to the 6-bp difference between the oranges and mandarins (Figure 5). The orange DNA solution was mixed with mandarin DNA solution at different ratios to run the parallel DGGE (Figure 6). Two banding patterns appeared in the polyacrylamide gel: the heteroduplex molecule type and the homoduplex one[25]. The heteroduplex molecule type of banding can be observed when there is more than one DNA type in the same PCR reaction. A heteroduplex has a mismatch in the DNA double-strand that causes a distortion in its usual conformation, which has a destabilizing effect and causes the DNA to denature at a low denaturant concentration. The migration of heteroduplex bands was slower than that of the corresponding homoduplex bands because the volume of heteroduplex molecules was larger than that of homoduplex molecules. The increase in ratio of mandarin to orange DNA concentrations ratio was accompanied by an increase in heteroduplex molecules. The concentration of heteroduplex molecules peaked when there was 25% orange DNA in the solution (Figure 6). It might be due to the mismatch of forward primer *trn*L3 in the

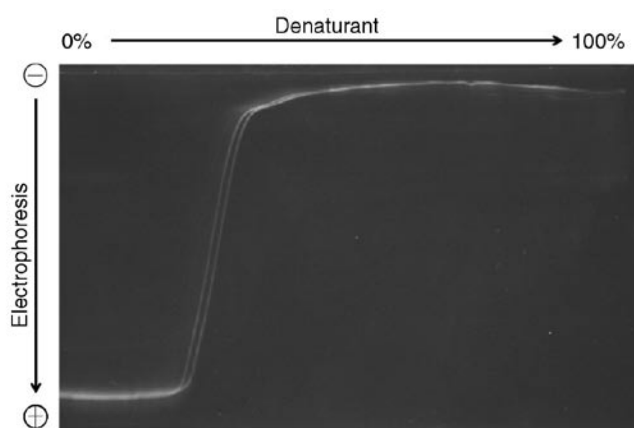

Figure 4. Perpendicular DGGE of orange and mandarin amplified by *trn*L3/*trn*F3 primer. The denaturant concentration of the 10% polyacrylamide gel is between 0 and 100%.

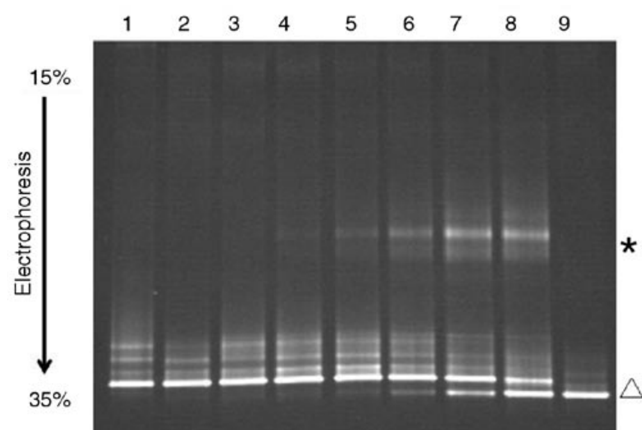

Figure 6. The parallel DGGE of orange DNA solution (O) which was mixed with mandarin DNA solution (M) at different percentage. Lane1: 100% O; Lane 2: 99.5% O + 0.5% M; Lane 3: 95% O + 5% M; Lane 4: 90% O + 10% M; Lane 5: 75% O + 25% M; Lane 6: 50% O + 50% M; Lane 7: 25% O + 75% M; Lane 8: 10% O + 90% M: Lane 9: 100% M. *The heteroduplex molecular. $^{\Delta}$The homoduplex molecular.

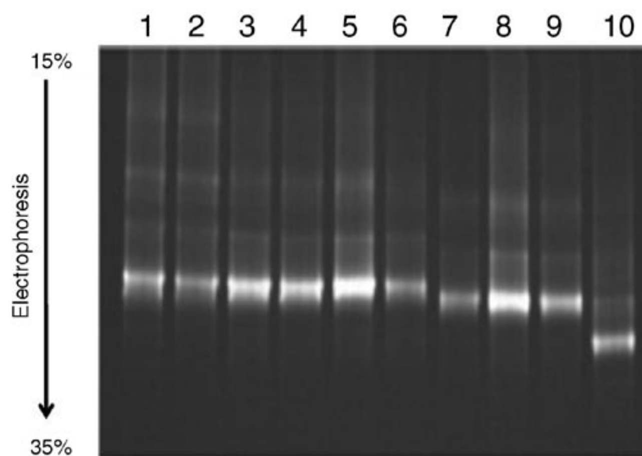

Figure 5. The parallel DGGE of six oranges and four mandarins. Lane 1: Liucheng; Lane 2: Pineapple; Lane 3: Valencia; Lane 4: Hamlin; Lane 5: Parson; Lane 6: Navel Seeding; Lane 7: Ponkan; Lane 8: Murcoot; Lane 9: Satsuma Seeding; Lane 10: Tankan.

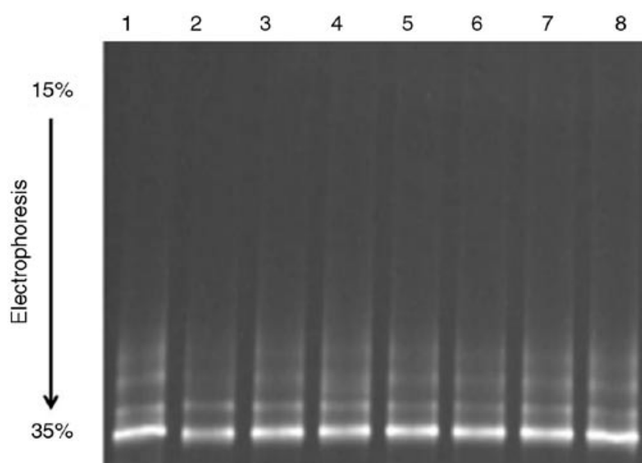

Figure 7. The parallel DGGE of Liucheng DNA solution (L) which was mixed with Navel Seeding DNA solution (N) in different percentage. Lane1: 100% L; Lane 2: 99.5% L + 0.5% N; Lane 3: 95% L + 5% N; Lane 4: 90% L + 10% N; Lane 5: 75% L + 25% N; Lane 6: 50% L + 50% N; Lane 7: 25% L + 75% N; Lane 8: 100% N.

mandarin *trn*L intron sequence, which affects the efficiency of primer. After analysis of the orange and mandarin DNA mixture, the same citrus species were mixed for the feasibility study. Figure 7 shows the parallel DGGE results for the orange DNA solutions mixed with other orange DNA solutions at different percentages. All samples showed only the homoduplex banding pattern. It was found that the heteroduplex molecule type of banding appeared only when the DNA sequences of samples were different.

III. *Detection of Adulteration in Commercial Orange Juice*

Eight commercial orange juice samples which declared to be 100% pure orange juices were employed to run the parallel DGGE for adulteration detection. There was more than one heteroduplex in the commercial samples (Figure 8). Two reasons might account for such finding. First, DNA of other additives present in the 100% orange juice samples was also amplified by the *trn*L3/*trn*F3 primer. Second, the 100% orange juice was mixed with several other citrus juices. However, since the specificity of the *trn*L3/*trn*F3 primer has been confirmed in this study, the identification of more than one heteroduplex in the samples must be attributed solely to the second reason. Commercial orange juices typically have other citrus juices added to increase the flavor and color as well as to promote quality and acceptability of 100% orange juice.

CONCLUSIONS

This study showed that the *trn*L intron of chloroplast DNA is a useful marker for differentiating oranges from mandarins. In previous studies, the primer pair was designed in variable sequences, and the results of these studies were determined by the appearance or absence of banding patterns. By adding the denaturing gradient to gel electrophoresis, the PCR products with the same fragment size and different DNA sequence can be identified in a single electrophoresis. This method is effective for purity detection as well as other processed citrus food products.

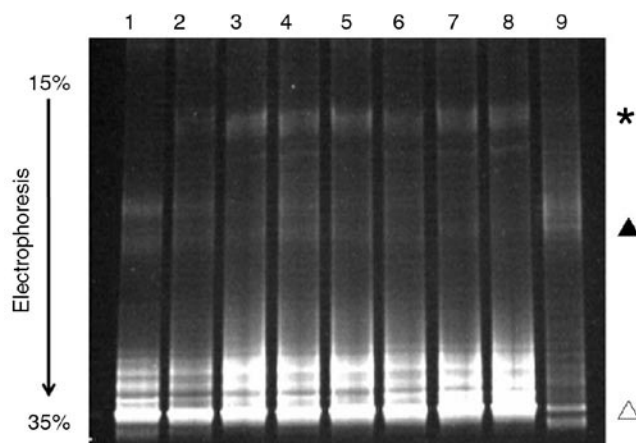

Figure 8. The parallel DGGE of 100% declared commercial citrus juice. Lane 1 to Lane 8: 100% declared commercial orange juice; Lane 9: orange DNA solution mixed with 10% mandarin DNA solution. *The heteroduplex molecular of orange DNA mixed with other unknown citrus DNA. ▲The heteroduplex molecular of orange DNA mixed with mandarin DNA. △The homoduplex molecular of orange or mandarin DNA.

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褪黑激素對於急性酒精作用PC12細胞株抗氧化狀態之影響

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摘　要

因過量酒精的消耗，可能增加活性氧的產生，進而造成組織的氧化性傷害。本實驗探討當PC12細胞株經急性酒精作用之後，褪黑激素（melatonin）對於抗氧化狀態的影響評估。在本研究中，我們先施予不同濃度的褪黑激素24 hr後，再以150 mM酒精作用4 hr；之後我們分析下列各項：細胞存活率（cell viability）、乳酸去氫酶釋出百分比（% lactate dehydrogenase released）、麩胱甘肽濃度（glutathione，簡稱GSH）、超氧陰離子歧化酶（superoxide dismutase，簡稱SOD）和麩胱甘肽還原酶（glutathione reductase，簡稱GRx）的活性及羰基化蛋白質濃度（protein carbonyl）等，以觀察其氧化性傷害與評估抗氧化狀態。結果顯示：當PC12細胞株經10、100與1000 μM褪黑激素處理後，在急性酒精作用組別中的細胞存活率及GSH濃度皆呈現增加現象，而酒精引起的細胞毒性及羰基化蛋白質濃度則呈現減少趨勢，但對於SOD活性則無影響。另外，10及100 μM褪黑激素對於GRx活性並無顯著差異，但在1000 μM的褪黑激素作用組別，GRx活性則呈現下降的現象。由上述結果顯示：褪黑激素可提升GSH濃度以降低急性酒精作用PC12細胞株引起的氧化性傷害，因此若給予適當的褪黑激素，可能可達到保護細胞的效果。

關鍵詞：PC12細胞株，酒精，褪黑激素，麩胱甘肽，羰基化蛋白質

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從雞卵伴白蛋白水解物分離之前驅藥型胜肽以抑制血管收縮素轉化酶的作用

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摘　要

從雞卵伴白蛋白的水解物所分離出一種對血管收縮素轉化酶（angiotensin I-converting enzyme，ACE）具有抑制作用的胜肽，並定出此一胜肽之胺基酸序列爲Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr。此一胜肽對ACE活性表現出濃度依存性的抑制效果；有效抑制濃度（IC_{50} value）爲102.8 μM。若將Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr與ACE反應後，所得到產物（Lys-Val-Arg-Glu-Gly-Thr）的有效抑制濃度則變爲9.1 μM；爲反應前的11倍。因此，Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr可視爲前驅藥（pro-drug）。將Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr與Lys-Val-Arg-Glu-Gly-Thr再分別經由尾部靜脈注射至高血壓大白鼠（SHR）體內並觀察其血壓的變化。結果發現，Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr於注射後40 min達到最高的降血壓值。可是，以相同的方法處理Lys-Val-Arg-Glu-Gly-Thr，卻於靜脈注射後20 min就觀察到最高的降血壓值；此種出現降血壓時間的20 min差異可能是Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr於動物體內與ACE反應，轉化爲活性型之抑制胜肽（Lys-Val-Arg-Glu-Gly-Thr）所需要的時間。因此，由以上結果可推論Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr爲一種前驅型（pro-drug）胜肽，於動物體內被分解爲Lys-Val-Arg-Glu-Gly-Thr來抑制ACE的活性。

關鍵詞：血管收縮素轉化酶抑制劑，降血壓胜肽，雞卵伴白蛋白

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兩種白毛藤之分析鑑定：白英和尋骨風

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摘　要

隨著越來越多人使用中藥，中藥鑒定日益成爲重要的國際性問題。使用品種錯誤的中藥將會導致新的疾病產生甚至死亡。中藥鑒定所要解決的問題主要是中藥的名稱混亂及不斷出現的僞品。2004年春香港發生一宗由於中藥異物同名造成的嚴重醫療事故，原因是具相同異名“白毛藤”的中藥白英和尋骨風在市場上混亂使用。白英基本上不具毒性，而尋骨風則含有馬兜鈴酸，不當使用能導致腎衰竭和尿道癌。本文對白英和尋骨風從植物形態、藥材性狀、顯微特徵及化學分析四方面進行系統之分析鑒定，爲澄清白英和尋骨風的混亂提供鑒別標準。

關鍵詞：馬兜鈴酸，白英，尋骨風，白毛藤

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利用變性梯度膠體電泳檢測柑橘果汁中之攙假

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摘　要

*trn*L內含子（*trn*L intron）及*trn*L-*trn*F非轉譯區（*trn*L-*trn*F non-coding region）近年來已成爲植物分類的重要工具。本實驗將*trn*L內含子及*trn*L-*trn*F非轉譯區用於分辨甜橙以及寬皮柑，發現這兩種柑桔在*trn*L內含子（*trn*L intron）之位置有序列上之差異，因此針對此差異設計引子對（primer pair）將其擴增，並將擴增結果進行變性梯度膠體電泳系統（denaturing gradient gel electrophoresis）分析。以此方法對8種市售宣稱100%甜橙果汁進行分析，結果發現全部皆攙有非甜橙類的柑桔果汁。由此可知，變性梯度膠體電泳分析確實可用於甜橙果汁攙假的檢驗。

關鍵詞：*trn*L，變性梯度膠體電泳系統（DGGE），柑桔，葉綠體