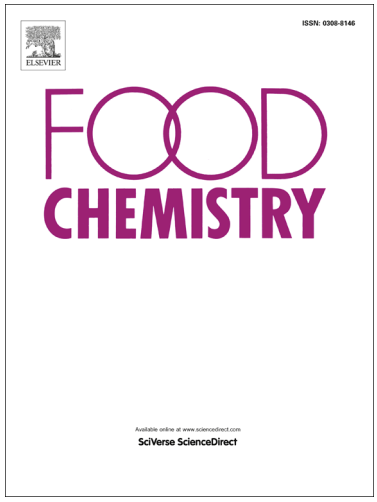

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Lemon Yellow #15 A New Highly Stable, Water Soluble Food Colorant from the Peel of *Citrus limon*

Xiaoyan Chen[a], Yuanqing Ding[b], Billy Forrest[c], Joonseok Oh[c], Stephanie M. Boussert[d], Mark T. Hamann[a,*]

[a]Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, Medical University of South Carolina, Charleston, SC 29425, USA; [b]National Center for Natural Products Research, Department of BioMolecular Sciences, Division of Pharmacognosy, and Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, Oxford, MS 38677;[c] Division of Pharmacognosy, School of Pharmacy, The University of Mississippi, Oxford, MS 38677[d] Department of Chemistry and Biochemistry, College of Charleston, Charleston, SC 29401, USA;

[*]Corresponding author: Mark T. Hamann,

70 President Street, Room 406 DDB, Charleston, SC 29425, USA

Phone: 843-876-2316

Email: hamannm@musc.edu

ABSTRACT

To provide stable and low-cost naturally derived yellow pigments, a variety of food byproducts were evaluated and the constituents of lemon peel have emerged yielding a highly promising natural product with applications as a food dye. Here we report a new, highly stable and water soluble food dye called yellow #15 from the ethanol extract of the zest of *Citrus limon*. The structure of lemon yellow #15 was carefully assigned on the basis of spectroscopic data, including 1D and 2D NMR spectroscopy, and the absolute configuration was established by comparison of the experimental CD with calculated electronic circular dichroism (ECD) spectral data. CIELAB values and Delta CIELAB were measured and revealed this new water-soluble pigment has superior light stability relative to other natural products used as food dyes.

Keywords: Lemon Yellow #15; food colorant; *Citrus limon*; 3-Hydroxy-3-methylglutaryl (HMG)

1. Introduction

The last few years have witnessed a movement by food companies to replace artificial dyes and preservatives with natural products. This movement has been in response to consumer demands for natural and safer plant-based food ingredients (Rozin, Spranca, Krieger, Neuhaus, Surillo, Swerdlin, et al., 2004; Sigurdson, Tang, & Giusti, 2017). Public opinion is that plant-based food additives present less danger than petroleum or synthetic derived ingredients and based on the structural features this is reasonable in many cases (Dickson-Spillmann, Siegrist, & Keller, 2011; Lalor, Madden, McKenzie, & Wall, 2011). This ideation has grown from an expanding number of reports of adverse reactions, toxicity, carcinogenicity, behavioral and neurocognitive effects related with synthetic food colorants and a substantial number of associated impurities (Amin, Abdel Hameid, & Abd Elsttar, 2010; Martins, Roriz, Morales, Barros, & Ferreira, 2016). The most commonly used synthetic yellow food dyes, FD&C yellow No.5 and FD&C yellow No.6, were implicated in a variety of adverse reactions including cytotoxicity, genotoxicity and carcinogenicity (Amchova, Kotolova, & Ruda-Kucerova, 2015; Mpountoukas, Pantazaki, Kostareli, Christodoulou, Kareli, Poliliou, et al., 2010; Soares, Araujo, Ramos, Pinto, Khayat, De Oliveira Bahia, et al., 2015). In 2009, European Food Safety Authority (EFSA) re-evaluated the safety of tartrazine (FD&C yellow No. 5) and concluded that the daily use of tartrazine within the Acceptable Daily Intake (ADI) of 7.5 mg/kg bw/day was safe (Amchova, Kotolova, & Ruda-Kucerova, 2015). In 2014, the ADI for sunset yellow (FD&C yellow No. 6) was raised to 4 mg/kg bw/day from a temporary ADI of 1 mg/kg bw/day that was established in 2009 (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2014). In the USA, Food and Drug Administration Food Advisory Committee reviewed the relationship between exposure to color additives and hyperactivity in children and did not find casual evidence to support the link

in normal consumption (Amchova, Kotolova, & Ruda-Kucerova, 2015). The current tendency of the food industry to voluntary use more naturally derived colors is due to consumer demands. In addition to increased safety, naturally derived colorants may in many cases possess desirable health benefits, which can be evidenced with the nature-derived dye, annatto. Annatto is a yellow–orange dye obtained from *Bixa orellana*, primarily composing of the carotenoids bixin and norbixin (Scotter, 2009), and has been shown to exert antioxidant, anti-carcinogenic, and anti-inflammatory properties in vitro and in rats (Shahid ul, Rather, & Mohammad, 2016). The administration of bixin was able to protect against the atherosclerotic lesion in hypercholesterolemic rabbits and stimulate immune response in dogs (Park, Mathison, & Chew, 2016; Somacal, Figueiredo, Quatrin, Ruviaro, Conte, Augusti, et al., 2015). Any therapeutic effects that colorants have are dependent on sufficient bioavailability through the diet and enhancing the bioavailability to improve biological efficacy of naturally derived colorants have been the subject of numerous investigations (McGhie & Walton, 2007; Stanic, 2017). The naturally-derived food colorants explored thus far fall primarily into five main groups including the anthocyanins, carotenoids, betalains, chlorophylls, and phenolic compounds (Rodriguez-Amaya, 2016). Some natural product groups have a long history of use in the food industry as well as a long history of use in the human diet (Bridle & Timberlake, 1997). Annatto, *β*-carotene, and turmeric are among the most commonly used yellow color additives approved by both European Food Safety Authority (EFSA) and US FDA (Wrolstad & Culver, 2012). However relative to their synthetic counterparts, application of naturally derived colorants in the food industry is still limited, especially considering the strong desire both by consumers and food manufacturers to replace artificial food additives with natural ingredients (Rodriguez-Amaya, 2016). The primary limitations include the instability and high cost of naturally derived colorants

(Delgado-Vargas, Jimenez, & Paredes-Lopez, 2000; Rodriguez-Amaya, 2016). For example, the major pigments of turmeric (E100), are curcumin, demethoxycurcumin, and bis-demethoxycurcumin. These pigments are susceptible to light, heat, oxygen and alkaline conditions that limit turmeric's use in foods (C. & R.W., 1996; D.M., C.R., & V.R.N., 2015). The poor water solubility of most current utilized naturally-derived colorants need them to be dispersed in other suitable matrices before they can be utilized to water based foods (Alison & Paul, 2000). It is important to emphasize that a colorant has to be approved for use in foods despite the artificial or natural source and the regulation varies among different countries, which gives rise to another challenge for a new emerging colorants (Magnuson, Munro, Abbot, Baldwin, Lopez-Garcia, Ly, et al., 2013).

The yellow pigments from lemon peel represents a potentially useful byproduct of lemon that could be isolated and prepared after the generation of juice and essential oils. This application may become more economically and environmentally sensible considering that up to 50 to 65% of the whole fruits remain as peel byproduct (Mandalari, Bennett, Bisignano, Saija, Dugo, Lo Curto, et al., 2006) and lemon processing totaled 2.88 million tons during the 2014-2015 season (United States Department of Agriculture, National Agricultural Statistics Service, 2015). In addition to lemon yellow #15 reported here, the lemon peel byproducts contain flavonoids including naringin, hesperidin, narirutin, neohesperidin, and related metabolites (Del Río, Fuster, Gómez, Porras, García-Lidón, & Ortuño, 2004; Miyake, Yamamoto, Morimitsu, & Osawa, 1998). Besides adding color, these flavonoids have been shown to possess health beneficial effects. These molecules are well known for antioxidant properties that have been evaluated extensively in vitro (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997; Miyake, Yamamoto, Morimitsu, & Osawa, 1997). The effectiveness of citrus flavonoids pertaining to

cancer prevention and anti-inflammation has been assessed in vitro and using animal studies (Manthey, Grohmann, & Guthrie, 2001). The intake of *citrus limon* peel extract provided protection from renal damage on experimentally induced urolithic rats (Sridharan, Michael, Arya, Mohana Roopan, Ganesh, & Viswanathan, 2016). To increase the quality of food and address customer demands for food safety by using natural, plant-based ingredients and additives, we carried out a project investigating colorants from fruits and vegetables. This investigation into the components of lemon peel was part of this project and aimed at discovery of new stable, water-soluble, and obtainable yellow colorants. Here we reported a structure that emerged during our study that showed great potential as a future colorant.

2. Material and Method

2.1 Raw Material.

Citrus limon was obtained from a local grocery store and the peel (flavedo) was removed, lyophilized and ground to 1mm particle size using a grinder. 132.9 g dried material was generated from 305.6 g raw peel.

2.2 Extraction and Isolation.

The dried and ground zest was extracted immediately with ethyl acetate, ethanol, and water in sonicator for one hour, successively. The yield for each solvent was calculated according to yield (%) = weight of extract / weight of dried peel: EtOAc 14.3%, EtOH 19.7%, and water 5.32%. Ethanol extract (5g) was subjected to column chromatography on C18 silica gel and eluted with a gradient of EtOH─H_2O (0%; 20%; 40%; 80%; 100%EtOH) to afford five fractions. Fraction B (600mg) was further separated on Sephadex LH-20 eluted with MeOH-H_2O (0%; 20%; 100% MeOH, 200mL per gradient) to afford 39 subfractions. Fraction B-36 and Fraction B-37 were combined and purified by preparative HPLC using gradient EtOH-H_2O (0-100%, 120m) and 0.1%

ethanoic acid as the mobile phase (13mL/min) to yield lemon yellow #15 (4 mg, tR 42 min). Precoated silica gel 60 F254 plates from Merck were used for TLC. HPLC was carried out with a Phenomenex Silica column (10 × 250 mm, 5 μm) for semipreparative injections with a Waters dual pump model 510 and a UV absorbance detector model 486 and with fraction collection. Sephadex LH-20 (GE), and C18 silica gel (40-63 μm, sorbent) were used for column chromatography. Spots were visualized by spraying with 10% H_2SO_4 in EtOH followed by heating.

2.3 The spectroscopic and physical data of lemon yellow #15.

The HRESIMS, ^{1}H NMR, ^{13}C NMR, HSQC (Heteronuclear Single Quantum Correlation), HMBC (Heteronuclear Multiple Bond Correlation), and IR spectra of lemon yellow #15 were collected and analyzed to identify the planar structure as limocitrol 3-*O*-6"-[3-hydroxyl-3-methylglutaryl)]-glucopyranoside (Supplementary Material). After the hydrolysis of lemon yellow #15, the D-configuration of glucose was determined by optical rotation. A β glucosidic linkage of the glucopyranosyl unit was assigned via the $^{3}J_{1",2"}$ coupling constant (6.0 Hz). HRESIMS was performed using a Bruker Impact II TOF spectrometer. NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer and IR spectra with a Bruker Alpha FT-IR spectrometer using Opus 6.5 software.

ECD calculations for two possible diastereomers were conducted to determine the absolute configuration at C-3‴. The calculated ECD spectrum of 3‴*S* diastereomer was consistent with the experimental spectrum, which determined the absolute configuration of lemon yellow #15 to be *S* (Figure 3). Therefore, lemon yellow #15 was deduced as limocitrol 3-*O*-6"-[(*S*)-3-hydroxyl-3-methylglutaryl)]-β-D-glucopyranoside (Figure 1). All calculations at quantum mechanics level were performed by using the Gaussian 09 software package. Experimental ECD spectrum was

acquired with an Olis CD spectrophotometer. The concentration of the sample was 0.32 mg/mL in methanol and the path length was 1 cm.

The optical rotation was measured with a Autopol V polarimeter. The UV spectrum was collected with a Cary 50 spectrophotometer.

Limocitrol 3-O-6''-[(S)-3-hydroxyl-3-methylglutaryl)]-β-D-glucopyranoside: amorphous yellow powder; $[\alpha]^{20}_{D}$ -8.8 (*c* 0.16, MeOH); UV λmax (log ε) 205.0 (4.15), 277.0 (3.79), 351.0 (3.67) nm; IR νmax 1714.96, 1649.52, 1558.14, 1427.32, 1282.43, 1021.85, 990.16 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) see Table S1; negative-ion HRESIMS $[M - H]^-$ m/z 681.16927 (calcd for $C_{30}H_{33}O_{18}$, 681.16724).

2.4 Acid Hydrolysis of lemon yellow #15.

Lemon yellow #15 (3 mg) was refluxed in 6% HCl (5.0 mL) at 80°C for 2 h. Each reaction mixture was extracted with $CHCl_3$ (3 × 6 mL), and the H_2O phase was dried using a N_2 stream. The residues were separately subjected to column chromatography over silica gel with EtOAc-MeOH-H_2O (7:5:1) as eluent to yield (+)-D-glucose. The solvent system EtOAc-MeOH-H_2O (7:5:1) was used for TLC identification.

2.5 Color Measurements.

Color measurements were carried out by the Cary 50 UV-vis spectroscopy using illuminant D65 and CIE standard observer 2°. Lemon yellow #15, curcumin, and crocin were dissolved in methanol in concentration of 769 ηg/mL, 40ηg/mL, and 48 ηg/mL, respectively. Spectral reflectivity curve of lemon yellow #15 is presented in Fig 1 (E) in the range of 830-360 nm. The three solutions were set on a window to expose to sunshine at room temperature (Image S1). One month later their color values were measured again. The glass vials were purchased from VWR (catalog number 470151-622 and 470157-602).

3. Results and Discussion

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is an important intermediate in various metabolic pathways like in the synthesis of cholesterol and other isoprenoids (Friesen & Rodwell, 2004). (*S*)-HMG is usually inferred as naturally occurring considering the stereospecific activities of enzymes (Van der Heijden, de Boer-Hlupá, Verpoorte & Duine, 1994; Bergot, Baker, Lee & Schooley, 1979). Tsunashi Kamo *et al.* developed a method by reduction and amidation to confirm absolute configuration of the HMG group in natural products (Hattori, Horikawa, Makabe, Hirai, Hirota, & Kamo, 2007). Here we employed electronic circular dichroism (ECD) computational approaches to establish the absolute configuration of HMG in lemon yellow #15 (Ding, Li, & Ferreira, 2009). Both *R*- and *S*- configurations at C3''' in lemon yellow #15 (denoted as (3'''*R*)**1** and (3'''*S*)**1**, respectively) were evaluated using a conformational search using the OPLS_2005 force field in the MacroModel software package of Schrodinger Suite with an energy window of 130 kJ/mol. Eighteen of 488 (3'''*R*)**1** and 15 of 510 (3'''*S*)**1** conformers within an energy cutoff of 20 kJ/mol were chosen for geometry optimization and harmonic vibrational frequency computations at the B3LYP/6-31G** level in the gas phase (Figure 2). Boltzmann conformational distribution was calculated using the electronic and zero-point energies provided the above mentioned optimization process (Table 1 and 2, respectively). The conformers (3'''*R*)1-8 and (3'''*S*)1-12 were found to be the respective major conformers (90.5 and 98.8% of the (3'''*R*)**1** and (3'''*S*)**1** configurations). Interestingly, the HMG in (3'''*R*)1-8 formed a (C5'''O)H···O(C8) hydrogen-bond from the front of A/C plane in (3'''*R*)**1** configuration, whereas such a bond in (3'''*S*)1-12 was found from the rear of A/B plane in (3'''*S*)**1**. The conformers (3'''*R*)1-7 and (3'''*S*)1-10 were calculated to possess only 9.2 and 1.2% of the (3'''*R*)**1** and (3'''*S*)**1** configurations, respectively, and the (C5'''O)H was found to form a hydrogen-bond with O(C7)

instead of O(C8) both from the front of A/C plane. Notably, the (C5'''O)H···O(C7) distance in conformer (3'''*S*)1-10 is 0.07 Å longer than that in (3'''*R*)1-7**,** whereas the (C5'''O)H···O(C8) distance in (3'''*S*)1-12 is 0.09 Å shorter than that in (6'''*R*)1-8. These structural and energetic differences indicate that HMG in both (3'''*R*)**1** and (3'''*S*)**1** favors the formation of the (C5'''O)H···O(C8) hydrogen-bond more than (C5'''O)H···O(C7) hydrogen-bond. Furthermore (3'''*R*)**1** prefers the generation of the hydrogen bond from the front of A/B plane whereas (3'''*S*)**1** prefers from the rear. The ECD spectra for the two enantiomers were computed at the B3LYP/6-31G** level in the gas phase, and only the weighted ECD of (3'''*S*)**1** matches the experimental ECD data of lemon yellow #15 (Figure 3). Exemplified by the predominant conformer (3'''*S*)1-12, the $n \rightarrow \pi^*$ electronic excitations at 361 and 303 nm contributed to the experimentally observed negative Cotton effect (CE) at the broad wavelength range of 400–291 nm, that at 266 nm corresponded to the low-amplitude positive CE at 290–273 nm, whereas the high-amplitude negative CE at 272–210 nm can be attributed to the excited states at 272, 229, 216, 214, 211, 209, 208, and 206 nm, respectively. This analysis unambiguously assigns the HMG as *S*-configuration, leading the full structure of lemon yellow #15 defined as limocitrol 3-*O*-6''-[(*S*)-3-hydroxyl-3-methylglutaryl)]-*β*-D-glucopyranoside.

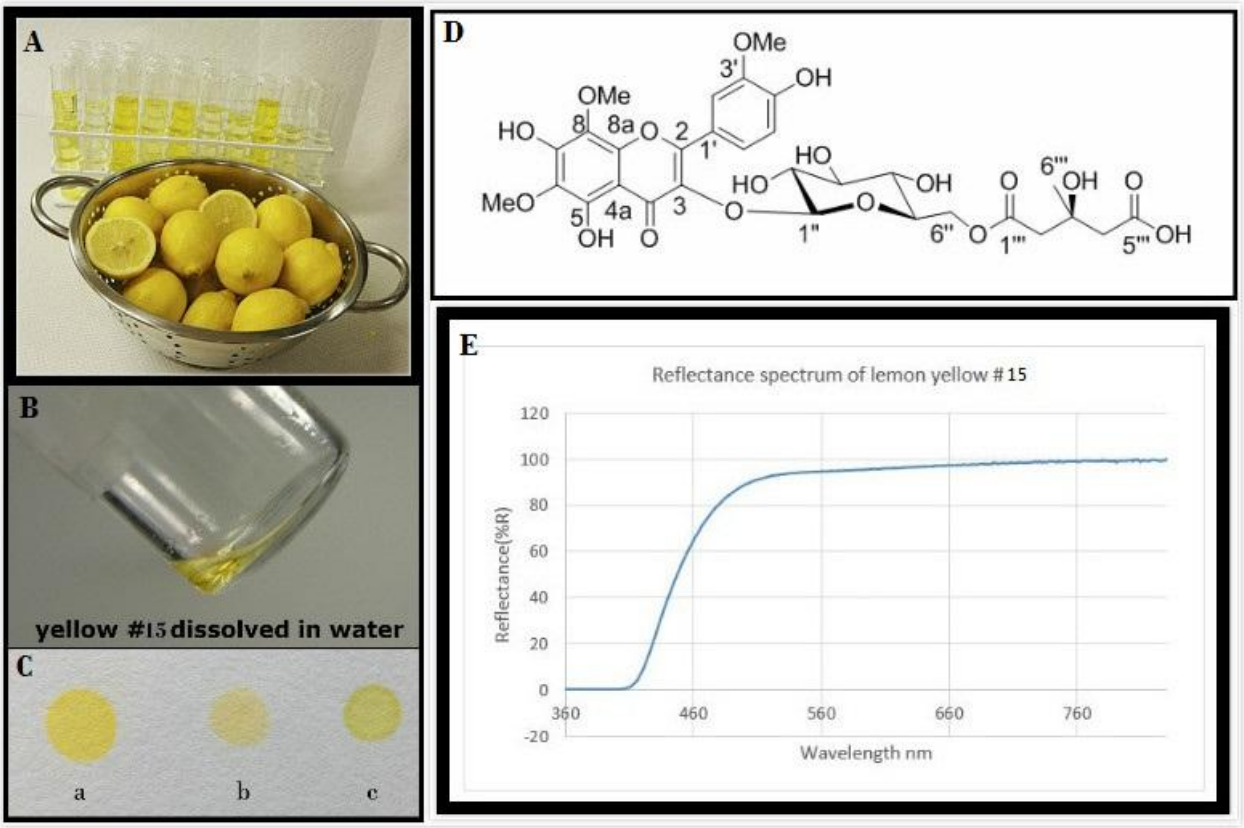

Figure 1. (A) Peel of *Citrus limon* was extracted for isolation of yellow pigment. (B) Pure lemon yellow #15 dissolved in water. (C) Filter paper dyed by curcumin (a), crocin (b) and yellow #15 (c) at 1 mg/ml, 2 mg/ml, 15 mg/ml separately in methanol considering curcumin is difficult to be dissolved in water. (D) Structure of lemon yellow #15. (E) Reflectance spectrum of yellow #15 solution 769 ηg/mL in methanol.

Table 1. Conformational Analysis of (3'''*R*) **1** at the B3LYP/6-31G** Level in the Gas Phase.

(3'''*R*)**1***	ΔE†	P% ‡	(3'''*R*)**1***	ΔE†	P% ‡
1	4.52	0.0	10	11.40	0.0
2	5.16	0.0	11	7.30	0.0
3	7.66	0.0	12	6.49	0.0
4	3.74	0.2	13	6.96	0.0
5	7.12	0.0	14	12.07	0.0
6	6.90	0.0	15	11.07	0.0
7	1.35	9.2	16	4.79	0.0
8	0.00	90.5	17	6.81	0.0
9	7.30	0.0	18	8.58	0.0

*Conformer number, † Relative energy (kJ/mol), ‡Conformational population.

Table 2. Conformational Analysis of (3'''*S*)**1** at the B3LYP/6-31G** Level in the Gas Phase.

(3'''*S*)**1***	ΔE†	P%‡	(3'''*S*)**1***	ΔE†	P%‡
1	12.51	0.0	9	5.57	0.0
2	7.11	0.0	10	2.60	1.2
3	17.50	0.0	11	15.44	0.0
4	7.02	0.0	12	0.00	98.8
5	15.76	0.0	13	7.34	0.0
6	12.45	0.0	14	12.04	0.0
7	10.50	0.0	15	6.68	0.0
8	20.70	0.0			

***** Conformer number, †Relative energy (kJ/mol), ‡ Conformational population.

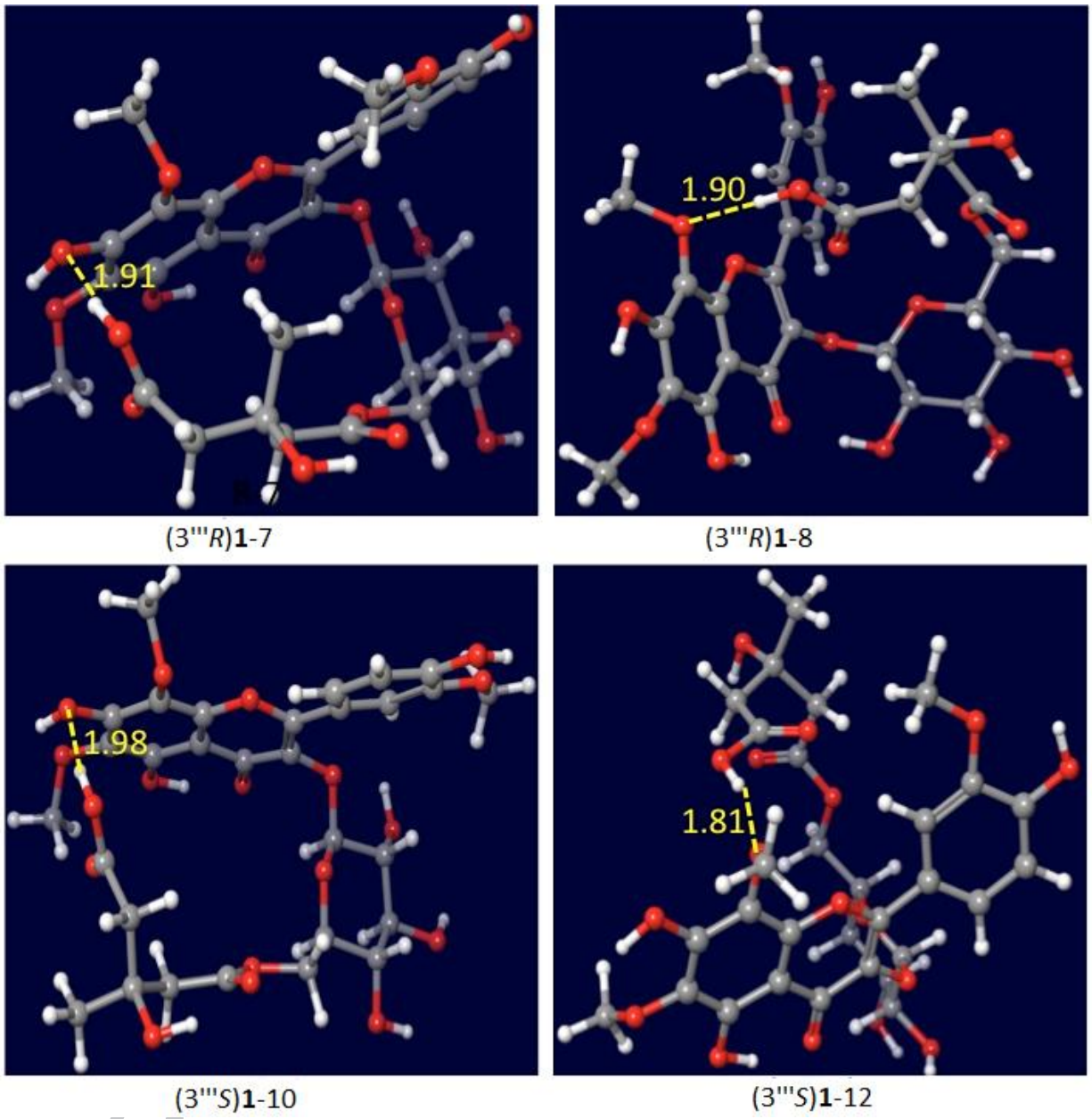

Figure 2. Optimized geometries of predominant conformers of lemon yellow #15 with *S*- and *R*-configurations at C3''' in the 3-hydroxy-3-methylglutaryl group (HMG).

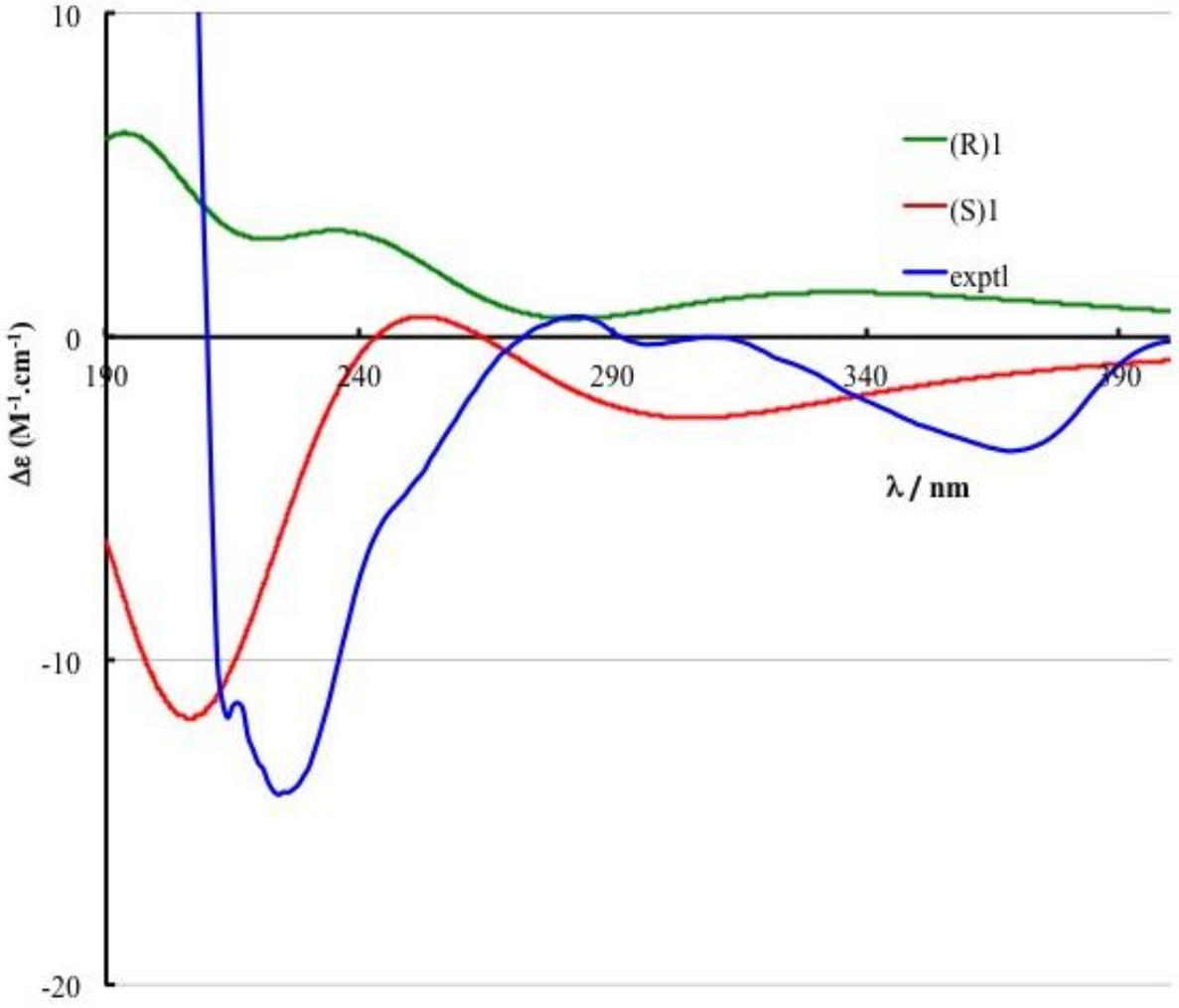

Figure 3. Experimentally observed ECD curve and weighted ECD spectra of lemon yellow #15 with *S*- and *R*- configurations at C3''' in the 3-hydroxy-3-methylglutaryl group (HMG).

What needs to be noticed is the correlation between anomeric hydrogen and C-3 can be observed in HMBC spectrum when the delay for evolution of long-range couplings was optimized to a J_{HC} of 4 Hz. If with J_{HC} =8 Hz or 12Hz, the correlation cannot be observed.

To assess the potential of lemon yellow #15 as a colorant, the color parameter values of it and two commercially available yellow colorants, curcumin and crocin, were measured (**Table 3**). *L** describes the lightness of the color, ranging from black (*L** = 0) to white (*L** = 100). *a** denotes the red (+)/green (-) value and *b** denotes the yellow (+)/blue (-) value (Zarena & Udaya Sankar,

2012). According to the *L** values, lemon yellow #15 has similar degree of lightness with the two commercial pigments. To test the stability of the three samples, the solution of lemon yellow #15, curcumin, and crocin were exposed to sunlight at room temperature for one month. Then these color parameter values were measured again and the total color changes were calculated from *L**, *a**, and *b** coordinates by means of the expression: $\Delta E^* = [(\Delta L^2)+ (\Delta a^2)+ (\Delta b^2)]^{1/2}$, which shows lemon yellow #15 is more stable than curcumin and crocin. In this study we found lemon yellow #15 is easy to dissolve in water, while curcumin and crocin showed lower solubility in water. And due to the low price and availability of lemon peel, lemon yellow #15 possesses a high probability to be developed as a yellow colorant for the food industry. This metabolite has been previously detected in the polar extract from lemon using MS/MS without the detailed assignment of NMR data, stereochemistry determination and color measurements (Ledesma-Escobar, Priego-Capote, & Luque de Castro, 2015).

Table 3. Colorimetric coordinates of lemon yellow #15 and commercial pigments[§]

sample	Time (weeks)	*L**	*a**	*b**	ΔE*
lemon yellow #15	0	97.2779	11.5872	32.2425	6.3
	4	97.5677	9.7207	26.2324	
curcumin	0	96.6245	20.7154	107.4093	101.3
	4	99.2772	2.6029	7.7897	
crocin	0	98.2925	18.4518	58.4755	25.0
	4	99.1405	13.1145	34.0024	

[§]For this colour measurement, lemon yellow #15**,** curcumin, and crocin were dissolved in methanol in concentration of 769 ηg/mL, 40ηg/mL, and 48 ηg/mL, respectively

4. Conclusion

There is a movement in the food industry to replace synthetic yellow food dyes with the naturally derived alternatives. However, the instability and high cost of existing natural product derived yellow colorants has hindered their utilization by the food industry. The new food dye, lemon yellow #15, was obtained from lemon peels and has revealed significant advantages as a food colorant. This compound possesses much greater light stability than curcumin which is the most stable natural dye of turmeric according to observable color changes with naked eye and the values of ΔE*. In addition, lemon yellow #15 is highly water-soluble, which makes its extraction, isolation and utility to food products more effective. Finally, the abundance of lemon peel, a byproduct of lemon juice production assures that its availability and production will be highly cost-effective.

Calculated electronic circular dichroism (ECD) was employed here and unambiguously determined the absolute configuration of the HMG group by comparison with the experimental CD, which is a convenient method and without loss of sample compared to the previous reported methods through making derivatives.

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Conflicts of Interest

The authors declare no conflict of interest.

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Highlights

- The new food dye, lemon yellow #15, was obtained from lemon peels and possesses much greater light stability than current natural-derived food dyes.
- Lemon yellow #15 is highly water-soluble, which makes its extraction, isolation and utility to food products more effective.
- The abundance of lemon peel, a byproduct of lemon juice production assures that its availability and production will be highly cost-effective.