Influence of Chemical Refining on the Major and Minor Components of Rice Bran Oil

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ABSTRACT: The effects of each individual step of the chemical refining process on major and minor components of rice bran oil were examined. In comparison with common vegetable oils, rice bran oil contains a significantly higher level of several bioactive minor components such as γ-oryzanol, tocotrienols, and phytosterols. Alkali treatment or neutralization results in a significant loss of oryzanol. In addition, it gives rise to a change in the individual phytosterol composition. After bleaching, some isomers of 24-methylenecycloartanol were detected. Because of their relatively high volatility, phytosterols and tocotrienols are stripped from the rice bran oil during deodorization and concentrated in the deodorizer distillate. At the same time, oryzanol is not volatile enough to be stripped during deodorization; hence, the oryzanol concentration does not change after deodorization. Complete refining removed 99.5% of the FFA content. Depending on the applied deodorization conditions, *trans* FA can be formed, but the total *trans* content generally remains below 1%.

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Rice (*Oryza sativa*) is the most important agricultural product in Asia, since it is the basic food for most of the population in this region. Rice milling yields 70% of rice (endosperm) as the major product, and by-products consist of 20% rice husk, 8% rice bran, and 2% rice germ. Rice bran can be used as feed or as a source of rice bran oil (RBO).

In comparison with most vegetable oils, RBO has a qualitatively different composition of bioactive minor components, such as γ-oryzanol, tocotrienols, and phytosterols (1). γ-Oryzanol derivatives in particular are found in only a very limited number of oils. γ-Oryzanol covers the whole group of ferulic acid esters of triterpene alcohols and phytosterols (2). The four major components of γ-oryzanol in RBO have already been identified as 24-methylenecycloartanol ferulate, campesterol ferulate, cycloartenol ferulate, and sitosterol ferulate (3–5). Vegetable oils are regarded as a rich natural source of dietary plant sterols. The majority of crude vegetable oils contain 1–5 g kg^{-1} of phytosterols, but RBO can contain up to 30 g kg^{-1} of phytosterols (2). The level of tocotrienols in RBO is also very high compared with other vegetable oils.

Because of the high level of active components, RBO is considered to be very health promoting. γ-Oryzanol and phytosterols have the capacity to lower blood cholesterol and decrease cholesterol absorption (6–8). Tocotrienols and γ-oryzanol are known as powerful antioxidants, and this is associated with the prevention of cardiovascular diseases and some cancers (1,9,10). Because of these beneficial effects, RBO has a high nutritional value and is therefore very appealing as a specialty oil in niche markets (1).

India and Thailand are the most important producers of RBO, together accounting for more than 225,000 metric tons of RBO per year. In the last several years, many RBO extraction plants have changed or improved their technology to become more efficient and to produce better-quality crude RBO. In addition, refining processes have been optimized to obtain high-quality RBO for human consumption (1). However, refining RBO is more complicated than refining other oils because of the difference in its composition of minor components (11).

The influence of refining processes on RBO has rarely been investigated. Yoon and Kim (12) briefly mentioned the effect of different chemical refining steps on the content of phosphorous, FFA, total sterols, total tocopherols, and γ-oryzanol. That report mainly described the oxidative stability of RBO. Krishna *et al*. (13) studied the effect of refining on the retention of γ-oryzanol in chemically and physically refined oil.

The aim of the current study is to give an overview of the effects of each individual step of the chemical refining process on the major and minor components of RBO. This paper includes the effect of chemical refining on several parameters such as the FFA content, color, FA composition, element content, and the amount and composition of minor components such as phytosterols, γ-oryzanol, tocopherols, and tocotrienols. Since most of the minor components are very beneficial for the health, it is considered important to know which stages of the chemical refining process cause significant losses or modifications in the composition of the minor components.

EXPERIMENTAL PROCEDURES

Samples. All the RBO samples were produced in the refining plant of the Thai Edible Oil Company (Bangkok, Thailand).

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The crude RBO was produced by using a continuous solvent extraction (hexane) process from Desmet Ballestra. This crude oil was neutralized with NaOH and then bleached with 2% of activated clay (WAC Classic 1G) at 105°C under reduced pressure. Dewaxing consisted of controlled cooling of the oil to 11°C, followed by filtration on a membrane press filter. The oil was then deodorized at 238°C at 6–7 mbar vacuum using 2.2% of steam for 90 min. The deodorizer was a continuous tray deodorizer (from Desmet) operating at a rate of 100 metric tons per day. All samples were stored at 4°C until analysis.

Materials. Standards of cholesterol (99+%) and squalene were purchased from Sigma-Aldrich (Bornem, Belgium). All solvents and reagents were of analytical grade and were purchased from Acros Organics (Geel, Belgium), VWR (Leuven, Belgium), or Sigma-Aldrich (Bornem, Belgium).

Analytical methods. The analyses were performed using the official methods of the AOCS unless otherwise stated (14). The FFA content was determined by titration following AOCS Official Method Ca 5a-40 (14). However in the case of RBO, the use of the indicator phenolphthalein leads to an overestimation of the FFA content because the phenolic hydroxyl group of the oryzanol causes a shift in the acid–base equilibrium. Therefore, an indicator with a different pH working range was selected, bromothymol blue.

The FA composition was analyzed according to AOCS Official Method Ce 1f-96 (14). The separation of FAME was performed on a CP-Sil 88 capillary column (15 m × 0.25 mm i.d., 0.20 μm film thickness; Varian-Chrompack, Sint-Katelijne-Waver, Belgium) in combination with a precolumn (0.53 mm i.d.) under the following conditions: oven temperature: 100–150°C (5°C/min), 150–170°C (1°C/min), 170–210°C (3°C/min); FID, 220°C; carrier gas, hydrogen (0.7 mL/min).

The elements P, Fe, Ca, and Mg were analyzed by inductively coupled plasma according to AOCS Method Ca 20-99 (14). The amounts of unsaponifiables and DG were determined by AOCS Methods Ca 6a-40 and Cd 11b-91, respectively (14). DG were analyzed by GC using a 15 m CP-Sil 8 LB-MS capillary column with 0.25 mm i.d. and 1 μm film thickness (Varian-Chrompack). On-column injection was used, and the analysis was performed under the following conditions: oven temperature: 60–140°C (30°C/min), 140–235°C (5°C/min), 235°C (7 min), 235–340°C (15°C/min), 340°C (15 min); FID, 360°C; carrier gas, hydrogen (1.38 mL/min). The level of γ-oryzanol was determined spectrophotometrically according to the method described by Seetharamaiah and Prabhakar (15). Tocopherol and tocotrienol contents were analyzed by normal-phase HPLC (Alltima Silica 5U, 250 mm × 4.6 mm i.d., particle size 5 μm; Alltech, Lokeren, Belgium) based on AOCS Official Method Ce 8-89 (16).

The method used for the analysis of total sterols in RBO was adapted from a method previously optimized by Verleyen *et al.* (17). Several changes were made because of the high concentration of unsaponifiable matter and phytosterols in RBO. Samples (0.5 g) were weighed and 5 mL of the internal standard solution (0.1 g/100 mL cholesterol in chloroform) was added. After evaporation of the chloroform, 6 mL of 6 M KOH in water and 10 mL of ethanol were added and the sample was saponified for 3 h at 75°C (reflux). After saponification, 10 mL of water was added and the unsaponifiables were extracted twice, the first time with 15 mL of petroleum ether and the second time with 15 mL of diethyl ether. The combined extracts were washed two times with 8 mL of a KOH solution (0.5 M in water) and two times with 8 mL of a NaCl solution (5% in water). Afterward, the extracts were dried, and after evaporation of the solvent, the residue was derivatized (80°C, 30 min) by dissolving it in 0.5 mL of dry pyridine and adding 1 mL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane as silylating agent. All samples were injected in the gas chromatograph within 6 h of preparation, and each sample was analyzed in duplicate. An EC5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Alltech) was used and 0.5 μL of sample was injected, with a split ratio of 1:10. The analysis was performed under the following GC conditions: oven temperature: 285°C (30 min), 285–300°C (10°C/min), 300°C (10 min); FID, 360°C; carrier gas, helium (0.5 mL/min).

The squalene content was analyzed using the same method as for the total sterol analysis. A calibration curve was made using the reference compound.

For identification of components of the unsaponifiable fraction, the silylated residue was analyzed using the GC–MS instrument equipped with an AT^{TM}-5ms column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Alltech). The following GC conditions were used: oven temperature: 285°C (20 min), 285–300°C (10°C/min), 300°C (10 min); carrier gas, helium (0.8 mL/min), split ratio, 1:10. The EI-MS conditions were as follows: ionization voltage, 70 eV; mass selective detector transfer line, 300°C; scan range, *m/z* 40–600 Thomson.

RESULTS AND DISCUSSION

An overview of the effect of each step of the chemical refining process on the quality of RBO is given in Table 1. As expected, neutralization decreased the FFA content considerably, from 7.53 to 0.12%. Furthermore, the P and Mg contents were significantly reduced by a factor of more than 100. As Table 1 shows, neutralization had the main bleaching effect. Crude RBO has a very dark color, and removing the color components is one of the critical aspects of refining.

After the final FFA stripping during deodorization, the fully refined oil had an FFA content of only 0.04%. The concentration of DG was constant (4%) across the consecutive refining stages.

Table 1 also shows that the FA composition did not change during neutralization, bleaching, and dewaxing. However, the high temperature of the deodorization process led to the formation of *trans* isomers, resulting in 0.8% of *trans* FA in the fully refined RBO.

Table 2 shows the composition of the unsaponifiable fraction at every step of the chemical refining process. Crude RBO

TABLE 1
Overview of the FFA, DG, and Element Contents, the Color Change, and the FA Composition During Chemical Refining of Rice Bran Oil

	Oil sample				
Parameters	Crude	Neutralized	Bleached	Dewaxed	Deodorized
FFA[a] (g/100 g)	7.53	0.12	0.19	0.19	0.04
DG (g/100 g)	3.9	4.0	4.1	3.9	4.0
Color					
1-in.	70Y/13R/6B	70Y/4.3R/2B	12Y/2R	—	—
5.25-in.	—	—	70Y/10R/0.2B	35Y/3.8R	13Y/2.2R
Minerals (ppm)					
P	488.30	3.90	0.10	0.20	0.20
Fe	5.30	0.30	0.30	0.01	ND[b]
Ca	27.40	1.20	0.40	0.10	0.10
Mg	118.10	0.90	0.10	0.01	0.03
FA[c] (%)					
14:0	0.4	—[d]	—	0.3	0.3
16:0	19.8	—	—	19.8	19.8
16:1	0.2	—	—	0.2	0.2
18:0	2.0	—	—	2.0	2.0
18:1	42.7	—	—	42.7	42.9
18:2*t*	ND	—	—	ND	0.5
18:2*c*	32.4	—	—	32.3	31.7
18:3*t*	ND	—	—	ND	0.3
18:3*c*	1.4	—	—	1.4	1.1
20:0	0.8	—	—	0.8	0.8
20:1	0.5	—	—	0.5	0.5
Total *trans*	ND	—	—	ND	0.8

[a]Expressed as oleic acid.
[b]ND, not detected.
[c]FA are designated by number of carbon atoms:number of double bonds.
[d]The FA composition of the neutralized and the bleached sample were not determined since no modifications were expected during these refining stages.

TABLE 2
Changes in the Composition of Unsaponifiable Matter During Chemical Refining of Rice Bran Oil

Parameters (g/100 g)	Crude	Neutralized	Bleached	Dewaxed	Deodorized
Unsaps[a]	5.4	4.2	3.8	3.6	2.7
Squalene	0.028	0.022	0.021	0.019	0.008
Total sterols	2.94	2.15	1.92	1.91	1.83
Total tocols[b]	0.078	0.080	0.078	0.078	0.062
Rest unsaps[c]	2.3	2.0	1.8	1.6	0.8

[a]Unsaps, unsaponifiable matter.
[b]Tocols, sum of tocopherols and tocotrienols.
[c]Rest unsaps = Unsaps–Squalene–Total sterols–Tocols.

contained 5.4% of unsaponifiable matter, consisting mainly of phytosterols[1], tocopherols, tocotrienols, squalene, and other nonpolar components. Among these nonpolar components, long-chain fatty alcohols (C_{22}–C_{34}) were detected in the crude, neutralized, and bleached samples and were identified by GC–MS. The concentration of squalene decreased after neutralization, and especially after deodorization. During this last processing step, squalene was partly stripped and recovered in the deodorizer distillate.

The total tocopherol/tocotrienol content was mainly affected by deodorization. A reduction of approximately 25% was observed after deodorization, which was largely due to the volatility of these components. The composition of the tocopherol fraction at each refining stage is given in Table 3. Through the refining process, the relative contents of γ- and δ-tocotrienols decreased, whereas the α-tocopherol and α-tocotrienol contents increased and the γ-tocopherol content remained constant.

Neutralization caused an important decrease in the γ-oryzanol content (Table 4). This observation is in agreement with the report of Krishna *et al.* (13). The loss of the γ-oryzanol into the soapstock also explains the decrease in the total sterols content. Bleaching and deodorization caused an additional loss of the total sterols content. During deodorization, some of the sterols were stripped into the deodorizer distillate. Moreover, both bleaching and deodorization can lead to the formation of steradienes. Ferrari *et al.* (18) also

[1]In this paper, "phytosterols" consist of the sum of 4-desmethylsterols, 4-methylsterols, and 4,4-dimethylsterols.

TABLE 3
Composition of the Tocopherol Fraction During Chemical Refining of Rice Bran Oil

Tocols (%)	Crude	Neutralized	Bleached	Dewaxed	Deodorized
α-Tocopherol	11.7	12.9	16.6	17.0	17.2
γ-Tocopherol	15.6	15.5	15.1	15.2	15.2
α-Tocotrienol	3.7	4.2	4.4	4.5	5.3
γ-Tocotrienol	66.7	65.4	62.6	62.5	61.8
δ-Tocotrienol	2.2	2.0	1.3	0.9	0.5

reported that deodorization is the principal step that increases the content of dienes. In fact, several steradienes were detected by GC–MS (but not quantified) in the fully refined RBO.

Identification of the different phytosterols was carried out by a combination of relative retention times and MS data (Table 5) of the trimethylsilyl derivatives, which were compared with data from the literature (4,5,19,20). The 4-desmethylsterols accounted for more than 60% of the total sterols, and within this group, the Δ^5-unsaturated 4-desmethylsterols formed the largest fraction. The main Δ^5-(4-desmethylsterols) present in RBO were β-sitosterol and campesterol. These sterols both have a saturated side chain and are therefore easily identified by MS. The major fragment ions were the ions at *m/z* 129 (TMSOH + C1–C3) and *m/z* (M − 129). Also, *m/z* (M − 90), corresponding to the loss of TMSOH, and the molecular mass ion (M) represented ions of relatively high abundance. It is more difficult to discriminate between the diunsaturated Δ^5-sterols that have an additional double bond in different places in the side chain, because all have the same M.W. However, the loss of typical fragments attributable to allylic cleavage of the side chain, in combination with relative retention times (RRT) known from the literature, made the identification of all Δ^5-diunsaturated sterols possible. The mass spectra of the Δ^7-sterols led to different fragmentation patterns. In comparison with the Δ^5-sterols, the ion at *m/z* 129 had a very low abundance or could not be detected at all, but in this case, the most abundant ions were those of the molecular ion and the ion at *m/z* 255. When an additional double bond was present, the allylic cleavage gave a different pattern, but in most chromatograms of the Δ^7-sterols, the ion at *m/z* 229 was characteristic. Moreover, it has been reported that the influence of the side chain on the RRT is the same for the Δ^5-sterols and the Δ^7-sterols (21). Therefore, the ratio of the RRT of the Δ^7-form and the Δ^5-form of sterols with the same side chain is constant. In the conditions used during the present investigation, the Δ^7-sterols always eluted 1.1–1.2 times later than their Δ^5-analogs. This is an important observation of great use, especially for identification of the diunsaturated sterols. The sterols with a saturated steroid skeleton always eluted directly after the corresponding Δ^5-sterols. During the present investigation, a trace of the ring-saturated form of avenasterol was detected and identified. So far, we have found no literature that confirms the presence of this phytosterol in vegetable oils. The identification of the 4-methyl and 4,4-dimethyl sterols was fully based on data reported in literature (4,5).

Normally, the relative proportions of the individual sterols do not change during the refining process (18). In the case of

TABLE 4
Content of Oryzanol and Sterols and the Sterol Composition of Rice Bran Oil Samples at Different Stages of Refining

	Oil sample				
	Crude	Neutralized	Bleached	Dewaxed	Deodorized
Oryzanol (g/100 g)	1.8	0.4	0.4	0.3	0.3
Total sterols (g/100 g)	2.94	2.15	1.92	1.91	1.83
Sterol composition (%)					
Campesterol + campestanol	14.6	11.2	11.1	10.5	10.6
Stigmasterol	6.1	6.6	6.6	6.0	5.8
Δ^7-Campesterol	1.0	1.2	1.0	1.3	1.2
Clerosterol	0.2	0.3	0.3	0.6	0.5
β-Sitosterol	27.8	30.3	32.6	32.5	31.7
Sitostanol + Δ^5-avenasterol	5.0	5.2	3.8	4.5	4.0
Δ^5(24)-Stigmastadienol	0.5	0.7	2.0	2.5	2.3
Gramisterol	1.9	3.8	3.2	2.3	3.1
Δ^7-Sitosterol + cycloartenol	11.7	11.5	11.3	10.9	10.9
Δ^7-Avenasterol	2.3	2.8	2.2	2.0	2.1
Isomer 24-methylenecycloartanol[a]	ND[b]	ND	0.7	0.9	0.9
24-Methylenecycloartanol	26.6	23.0	16.3	14.3	15.6
Citrostadienol	2.2	3.5	4.0	5.1	5.0
Isomer 24-methylenecycloartanol	ND	ND	4.8	6.5	6.3

[a]This includes the sum of three different isomers that elute before 24-methylenecycloartanol.
[b]ND, not detected.

TABLE 5
MS Data of Sterols in Rice Bran Oil

Sterol	RRT[a]	Major ions in mass spectra
Campesterol	1.20	472 (50), 457 (16), 382 (88), 367 (43), 343 (100), 261 (14), 255 (19), 213 (12), 129 (85)
Campestanol	1.22	474 (55), 459 (74), 417 (22), 384 (28), 369 (49), 306 (31), 215 (100), 75 (98)
Stigmasterol	1.27	484 (61), 394 (60), 379 (22), 355 (29), 255 (63), 159 (33), 129 (87), 83 (100)
Δ^7-Campesterol	1.33	472 (100), 457 (25), 386 (12), 367 (15), 255 (75), 229 (18), 213 (28), 75 (39)
Clerosterol	1.37	484 (53), 469 (15), 394 (42), 388 (18), 379 (17), 355 (31), 343 (38), 283 (35), 255 (24), 213 (26), 129 (100)
β-Sitosterol	1.41	486 (54), 471 (17), 396 (97), 381 (43), 357 (100), 255 (19), 129 (76)
Sitostanol	1.45	488 (60), 473 (69), 431 (21), 398 (28), 383 (50), 357 (27), 305 (30), 257 (12), 215 (100), 147 (30), 75 (97)
Δ^5-Avenasterol	1.46	484 (17), 469 (12), 386 (100), 296 (63), 281 (42), 257 (28), 129 (68)
Δ^5(24)-Stigmastadienol	1.53	484 (67), 469 (32), 394 (40), 386 (100), 371 (37), 355 (13), 343 (85), 296 (52), 281 (23), 257 (30), 253 (20), 129 (81)
Gramisterol	1.56	484 (13), 469 (16), 400 (19), 393 (11), 379 (12), 357 (100)
Δ^7-Sitosterol	1.58	486 (100), 471 (24), 381 (14), 343 (10), 255 (72), 229 (17), 213 (28)
Cycloartenol	1.59	498 (3), 483 (10), 408 (100), 393 (99), 365 (86), 339 (45), 69 (79)
Δ^7-Avenasterol	1.62	484 (3), 386 (48), 371 (7), 343 (100), 255 (10), 253 (14), 213 (13), 75 (25)
Isomer 24-methylenecycloartanol	1.70	512 (1), 497 (8), 422 (100), 407 (93), 379 (97), 353 (38), 325 (18), 300 (18)
24-Methylenecycloartanol	1.76	512 (3), 497 (10), 422 (100), 407 (99), 379 (100), 353 (29), 300 (17)
Citrostadienol	1.87	498 (5), 483 (8), 400 (58), 393 (6), 357 (100)
Isomer 24-methylenecycloartanol (cyclobranol)	1.93	512 (4), 497 (10), 422 (100), 407 (95), 379 (91), 353 (38), 339 (15), 300 (8), 297 (12)

[a]RRT, retention time relative to cholesterol.

RBO, there was a clear difference in the composition of crude and neutralized oil. The relative amounts of campesterol and 24-methylenecycloartanol were higher and β-sitosterol was lower in the crude sample compared with the neutralized sample. This result suggests that campesterol and 24-methylenecycloartanol were relatively more present as ferulic acid esters (oryzanol) than were β-sitosterol or other phytosterols.

This intensive study of the composition of the individual phytosterols during the chemical refining process also showed that 24-methylenecycloartanol underwent isomerization, mainly owing to the neutralization and bleaching steps. This phenomenon has been reported before in relation to the influence of the hydrogenation process on C24-methylene and C24-ethylidene sterols (22). The formation of unknown components, together with a significant reduction of the 24-methylenecycloartanol content, was confirmed by GC–MS. Different reactions can lead to the formation of these structural isomers, e.g., geometrical and positional isomerization of the double bonds in the side chain and the opening of the cyclopropane ring. Consequently, a whole range of components can be formed during refining. In the present investigation, four different isomers of 24-methylenecycloartanol were detected. Three of the four components eluted before 24-methylenecycloartanol, and the most abundant isomer eluted after citrostadienol. Based on the report by Strocchi and Marascio (22) and the study of the GC–MS spectrum, this compound could be identified as cyclobranol. This is an isomer formed because of the positional isomerization of the double bond in the side chain from the C-24(28) to the C-24(25) position. Cyclobranol represents 6.3% of the sterols of the fully refined oil, whereas it was not detected in the crude RBO.

Table 6 shows the composition of the RBO deodorizer distillate. The deodorizer distillate was clearly composed mainly of FFA and unsaponifiable matter. A low valorization of the deodorizer distillate as a vitamin E source was expected, as the tocopherol and tocotrienol contents were rather low. On the other hand, the level of phytosterols was quite high (14.8%). The sterols profile of the distillate differed totally from that of the RBO. The common Δ^5-unsaturated 4-desmethylsterols (campesterol, stigmasterol, and β-sitosterol) were present in

TABLE 6
Composition of the Rice Bran Oil Deodorizer Distillate

Parameters (g/100 g)	Deodorizer distillate	
FFA[a]		32.9
MG		5.8
DG		6.1
Unsaponifiable matter		37.9
Squalene		1.9
Composition tocols[b]	g/100 g	%
α-Tocopherol	0.6	17.5
γ-Tocopherol	0.5	16.8
α-Tocotrienol	0.1	4.2
γ-Tocotrienol	1.9	60.4
δ-Tocotrienol	0.0	1.2
Total tocols	3.2	100.0
Composition sterols	g/100 g	%
Campesterol + campestanol	2.11	14.3
Stigmasterol	1.80	12.1
β-Sitosterol	6.16	41.5
Sitostanol + Δ^5-avenasterol	0.48	3.3
Δ^5(24)-Stigmastadienol	0.51	3.4
Gramisterol	0.12	0.8
Δ^7-Sitosterol + cycloartenol	0.65	4.4
Δ^7-Avenasterol	0.06	0.4
Isomer 24-methylenecycloartanol[c]	0.51	3.4
24-Methylenecycloartanol	1.31	8.9
Citrostadienol	0.56	3.8
Isomer 24-methylenecycloartanol	0.54	3.7
Total sterols	14.83	100.0

[a]Expressed as oleic acid.
[b]Tocols, sum of tocopherols and tocotrienols.
[c]This includes the sum of three different isomers that elute before 24-methylenecycloartanol.

much higher levels and accounted for almost 70% of the total sterols fraction. This could be explained by the fact that these sterols are present in a higher concentration in the free form or by a difference in volatility.

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