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Scalable Preparation of High Purity Rutin Fatty Acid Esters

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Abstract Investigations into expanded uses of modified flavonoids are often limited by the availability of these high purity compounds. As such, a simple, effective and relatively fast method for isolation of gram quantities of both long and medium chain fatty acid esters of rutin following scaled-up biosynthesis reactions was established. Acylation reactions of rutin and palmitic or lauric acids were efficient in systems containing dried acetone and molecular sieves, yielding from 70–77% bioconversion after 96 h. Thereafter, high purity isolates (>97%) were easily obtained in significant quantities following a two-step solvent purification procedure whereby excess fatty acid substrate was first removed in a heptane/water (4:1, v/v) system, followed by selective ester extraction using an ethyl acetate/water system (1:6, v/v) at elevated temperature.

Keywords Biosynthesis · Flavonoids · Rutin ester · Enzymatic esterification · Scalable preparation · Solvent purification system

Introduction

As secondary plant metabolites, flavonoids are found in a broad range of foods including green and black teas, apples, plums, strawberries, asparagus, citrus fruits and cocoa. They have been linked to a wide range of health benefits, from the prevention and treatment of cardiovascular [1] and liver diseases to inhibitory effects on cancers [2]. In fact, anti-allergenic, anti-viral, anti-microbial, anti-mutagenic and anti-inflammatory properties have all been documented for flavonoids [3–7], yet it is their anti-oxidative related properties which have really generated the most interest to date.

In general, glycosylated flavonoids are hydrophilic bioactive compounds. It is generally recognized that application of these hydrophilic compounds in food systems as well as other areas (i.e., pharmaceuticals, cosmetics) could be easily expanded through improved solubility and miscibility properties in more hydrophobic environments [6, 8, 9]. To achieve this, one effective modification strategy involves introducing hydrophobic groups onto the flavonoid molecule through lipase-catalyzed esterification/transesterification reactions. To date, hydrophobic groups with chain lengths ranging from short (C2) to very long (C18) have reportedly been used in acylation reactions, with the quickest reaction times and highest yields generally reported for flavonoids acylated to shorter chain length substituents [10, 11].

Research aimed at developing new and improved enzymatic methods for production of flavonoid esters is ongoing, as is assessment of the resulting bioactive compounds for a wide range of applications [5, 12, 13]. Still, in order to evaluate and exploit the potential of these novel compounds fully, it is extremely important that they be available in large enough quantities and high enough purities for such testing. Simple isolation methods which can effectively extract the desired flavonoid esters from more complex reaction mixtures are absolutely preferred, but are often lacking. This leaves more complicated

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chromatographic fractionation (or else low time-productivity efficient TLC separation) as the only choice for isolation of these products from their structurally similar substrates. As such, the present investigation addressed the development of a simple, fast and efficient solvent purification alternative which can be used for the isolation of larger quantities of high purity fatty acid esters of rutin, following scaled-up batch enzymatic acylation reactions in an organic solvent medium.

Experimental

Materials

Commercially immobilized lipase from *Candida antarctica* (Novozym 435, with activity of 10,000 Propyl Laurate Units (PLU) per gram) was obtained from Novozymes A/S (Bagsværd, Denmark). Acetone (99.8%), acetonitrile (>99.9%), heptane (99%), ethyl acetate (99.8%), acetic acid (99.8%), activated molecular sieves (3Å), palmitic acid (>97%), and rutin hydrate (95%) were purchased from Sigma–Aldrich (Brøndby, Denmark). Lauric acid (<99%) was from Fluka (Buchs, Switzerland). Toluene (≥99.5%), petroleum benzene (60–80 °C range), iso-amyl alcohol (>99%) and dimethylsulfoxide (99.5%, DMSO) were from Merck (Darmstadt, Germany). Chloroform (HPLC grade) was from Lab-scan (Dublin, Ireland). Water employed for HPLC analysis was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system.

Production of Rutin Esters

Production of rutin esters was carried out through enzymatic esterification of rutin with either palmitic or lauric acids. Rutin (10–30 mM) and fatty acid (40–120 mM, rutin: fatty acid molar ratio 1:4) substrates were first dispersed in a reaction vessel containing a known volume of dried acetone (300 mL). Enzymatic synthesis was initiated by the addition of Novozym 435 lipase (20 g/L). Activated molecular sieves (100 g/L) were also employed for water removal in order to drive the reaction towards biosynthesis. Reactions were incubated at 50 °C with agitation (200 rpm) for up to a maximum of 144 h. During this time, reaction bioconversion was monitored periodically by HPLC to confirm production, with bioconversion (%) calculated following HPLC analysis as the area of the ester product divided by the total area, multiplied by 100. Overall, results reported in this study were based on HPLC–UV detection, unless otherwise stated.

Solvent Purification of Ester Products

Optimization of Extraction Conditions

Following palmitoyl rutin ester production, immobilized lipase and molecular sieves were filtered from the mixture, acetone was removed and the excess unreacted palmitic acid was extracted using heptane. The resulting rutin/ester mixture was then dried and used to investigate the extraction efficiency of six different solvents towards the ester product. Specifically, 10 mL solvent, 20 mL water and powdered rutin/ester mixture (3.3–6.1 mg) were added together, shaken vigorously and allowed to separate into phases. Samples were then removed and subject to HPLC–UV analysis to determine the extent of extraction. Extraction efficiency (%) of each solvent was calculated based on the amount of palmitoyl rutin ester extracted into the organic phase, calculated as the palmitoyl rutin ester area divided by the total area, multiplied by 100.

Optimized Isolation Procedure

Enzymatic reactions were halted by filtering off the lipase (along with the molecular sieves) from the reaction mixture. Acetone was removed by vacuum rotary evaporation and the solid mixture was divided between 2–3 centrifuge tubes where the unreacted palmitic acid was extracted through repeated (i.e., 3–4) washings with heptane using heptane/water (4:1, v/v) at room temperature. When particulate matter or foam was present, samples were centrifuged briefly (1–2 min, 2,800 rpm) before removal of the heptane layer. Next, rutin was separated from its fatty acid ester through solvent extraction using ethyl acetate/water (1:6, v/v) at 60 °C, whereby rutin was extracted into the water phase and its fatty acid ester was present in the ethyl acetate phase. Again, centrifugation was employed to speed phase separation (and foam reduction) following vigorous shaking. Extractions were repeated several times (i.e., >5) to allow particulate matter to be selectively solubilized into either the aqueous or organic phase. The ethyl acetate phase (dark yellow liquid with no particulates present, approx. 300–400 mL) was then dried by rotary evaporation, followed by a gentle stream of nitrogen. Fatty acid esters of rutin were later analyzed for purity.

Purity of the isolates was calculated following HPLC-ELSD analysis, as the ester product area/total area, multiplied by 100. Assuming a 1:1 reaction, multiplying initial rutin amount (mol) by reaction bioconversion yielded the amount of rutin ester produced; moreover, multiplying this value by the MW of the ester product resulted in the expected mass of the ester product. The extraction yield was calculated as the final mass of ester

product/expected mass of ester, multiplied by purity and expressed in percent. All reactions and preparations were carried out in duplicate, reported as the mean ± standard deviations.

Characterization of Ester Products

HPLC Analysis

HPLC analysis was carried out using an Agilent HPLC system (1100 series, Agilent Technologies, Germany) and an Ascentis RP-C8 column (5 µm, 250 × 4.6 mm, Sigma–Aldrich, St. Louis, MO). This system was equipped with an autosampler, on-line degasser, column heater, ultraviolet diode-array detector (UV-DAD) and evaporative light scattering detector (ELSD, PL-ELS 2100, Polymer Laboratories, Sweden) with computerized data handling and integration analysis (Majestix, Rev. B.01.01, Hewlett Packard). Injection volume was 10 µl. Water and acetonitrile (both containing 0.1% acetic acid) were employed as mobile phases A and B, respectively, in the following gradient elution: 10 to 100% B over 15 min, 100% B for 10 min, followed by 5 min re-equilibration time between samples. Column temperature was 40 °C, flow rate was 0.800 mL/min and detection was carried out either by UV at 280 nm and/or by ELSD using evaporator and nebulizer temperatures of 90 and 50 °C, respectively, and a nitrogen gas flow of 1.2.

ESI-TOF–MS Analysis

Following HPLC separation (Dionex Ultimate 3000) using a procedure modified slightly from the one detailed above (i.e., reduced flow of 0.200 mL/min, RP-C18 column (Atlantis T3, 3 µm, 150 × 2.1 mm, Waters, MA) and use of methanol as mobile phase B), reaction products were analyzed using an electrospray ionization (ESI) source coupled to a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-Q, Bremen, Germany). Ionization was performed in the negative mode with an 8 L/min nitrogen flow, 0.8 bar nebulizer pressure and a temperature of 190 °C. Scan range was from 50–1,200 *m/z*.

Results and Discussion

The efficient production and isolation of modified bioactive compounds is of particular concern for those wishing to probe their extended properties and improve our understanding of how these compounds actually work. To this end, it is important that these novel compounds be of high purity and easily available in quantities suitable for investigations.

Production of Rutin Esters

Lipase-catalyzed esterification/transesterification reactions used to produce modified flavonoid esters, such as the palmitoyl rutin ester (Fig. 1), are typically carried out in

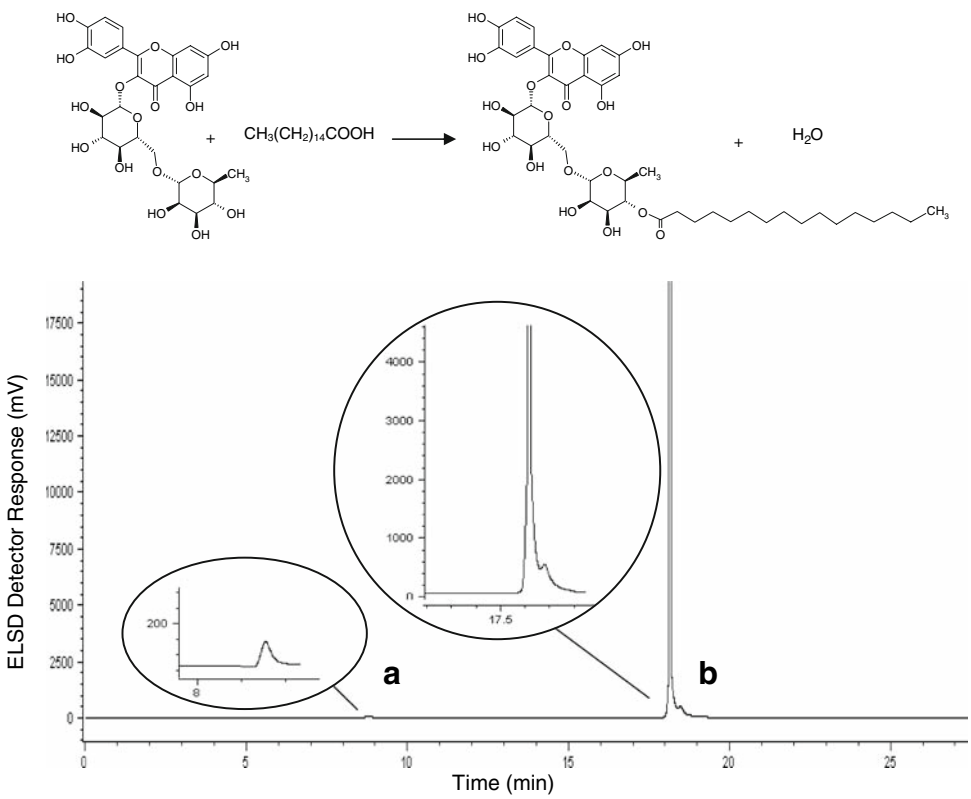

Fig. 1 HPLC–ELSD profile of palmitoyl rutin ester product following biosynthesis and isolation using a two-step extraction process; peak *a* identified as residual rutin substrate (<1%) and peak *b* identified as palmitoyl rutin ester product (>99%). Note the biosynthesis reaction of rutin with palmitic acid (ratio 1:4) was catalyzed by Novozym 435 lipase at 50 °C and 200 rpm in acetone (>96 h). HPLC analysis was carried out on a RP-C8 column, 0.800 mL/min flow, by gradient elution using acidified water and acetonitrile. ELSD settings for evaporator, nebulizer and gas flow corresponded to 90 °C, 50 °C and 1.2, respectively

organic solvent media and require several days to reach equilibrium [14, 15]. One of the major considerations when setting up this type of biosynthesis reaction involves solvent selection, as the number of organic solvents capable of solubilizing adequate amounts of both polar flavonoids and non-polar long chain fatty acid substrates is limited. An equilibrium bioconversion of 72% after 145 h was previously reported by Ardhaoui et al. for the acylation reaction of the hydrophilic flavonoid, rutin (log $P_{(calculated)} = -1.97$) with palmitic acid (log $P = 7.1$) in 2-methyl-2-butanol [14, 16, 17]. Similarly, acylation of rutin with oleic acid (log $P = 7.7$) in solvents such as acetonitrile, 2-methyl-2-butanol and acetone yielded from 45–71% bioconversion after 96 h [15, 17].

The solubility of rutin is generally low in most enzyme-compatible organic media; in fact, low rutin solubility in more polar solvents has been attributed to the presence of two sugars units on the rutin molecule [16]. However, a balance must be achieved which considers substrate solubilities and related equilibria, as well as reaction rates. Chebil et al. [16] previously reported rutin solubility at 50 °C in such promising reaction solvents as acetonitrile (0.50 mM), acetone (13.50 mM) and 2-methyl-2-butanol (60.03 mM). Such data coupled with literature reports detailing higher bioconversion yields for acylation reactions of rutin in acetone (1.6×) compared with 2-methyl-2-butanol [15], as well as the potential for facilitated purification due to acetone's significantly lower boiling point, contributed to its selection as reaction medium. Thus, a reaction system employing acetone was set up for the production of both medium and long-chain fatty acid esters of rutin whereby conditions such as high reaction temperature (50 °C), favorable substrate ratios (1:4) and low water activity (A_w) were selected to promote increased reaction rates and favor the production of ester products.

Acylation reactions in organic media typically employ rutin concentrations up to 16.5 mM [14, 15, 18]. In the present system, initial concentrations of rutin substrate were somewhat higher, ranging from 10–30 mM while the reaction itself was scaled-up significantly to 300 mL batch reactions. HPLC analysis confirmed equilibration of the palmitoyl rutin reaction system after approximately 96 h, as ester yields of 70, 77 and 76% were determined after 96, 120 and 144 h, respectively. Moreover, the biosynthesis reaction of rutin with lauric acid resulted in similar yields and equilibration times (Table 2). The corresponding concentration of rutin ester (produced per unit volume) approached a maximum of 19.61 g/L for the biosynthesis reaction of rutin with palmitic acid and 17.13 g/L for the reaction of rutin with lauric acid. These values far exceed amounts calculated for similar reactions (<8 g/L) in a range of media [10, 11, 14, 15, 18], due largely to careful selection of reaction parameters combined with increased rutin concentrations whereby gradual solubilization into the acetone media was observed over time.

Isolation of Ester Products

Biocatalyst and molecular sieves were filtered out of the system and the acetone was removed through rotary evaporation. The palmitoyl rutin ester reaction mixture was then subject to liquid extraction at room temperature using a water/heptane mixture (2/3, v/v), whereby palmitic acid was successfully extracted into the organic phase. Following palmitic acid removal, residual rutin was to be extracted into the water phase at elevated temperature (60 °C, 20 min agitation) using the same solvent system (water/heptane, 2/3, v/v) [11]. Unfortunately, HPLC analysis confirmed that the second step of this extraction process was not sufficiently selective to separate rutin and its palmitoyl ester, as both components were found present in high levels in the water phase. A more effective system was therefore needed in order to separate these two relatively similar molecules.

In total, six organic solvents were assessed at room temperature for their ability to extract palmitoyl rutin ester from an aqueous mixture containing rutin. As shown in Table 1, four of the six solvents tested were unable to extract the palmitoyl ester (or rutin for that matter) to any degree; this indicated that the solubility of the ester compound was not compatible with those particular four solvents. In contrast, iso-amyl alcohol was reasonably efficient (70%) while ethyl acetate proved to be an excellent choice (95.4%) for palmitoyl rutin ester extraction. At the same time, water phases employed in conjunction with these promising organic solvents contained varying levels of rutin, but no palmitoyl ester product. Investigations by Zi et al. [19] reveal a trend of increasing rutin solubility with increasing temperature for a range of organic solvents, with the exception of ethyl acetate. As the solubility of rutin in water also increases with temperature, extraction temperature was raised in order to decrease rutin solubility in ethyl acetate and further increase the selectivity of the extraction system. Results (Table 1) confirm that increasing the temperature of the extraction improves palmitoyl rutin ester extraction efficiency into the ethyl acetate phase.

Ethyl acetate has been used before for extraction of similar compounds [20]. However, introducing a water phase and regulating the extraction temperature allowed for selective removal of rutin from its palmitoyl ester product without the need for further chromatographic

Table 1 Extraction efficiency of selected solvents in a biphasic system, employed for the separation of palmitoyl rutin ester from its flavonoid (rutin) substrate following enzymatic ester production

Extraction efficiency (%)							
Solvent	Log *P*	Organic phase[a]			Water phase[b]		
		RT	50 °C	60 °C	RT	50 °C	60 °C
Heptane	4.0	0	0	0	+	+	++
Toluene	2.5	0	0		+	+	
Chloroform	2.0	0	0		+	+	
Petroleum benzene	2.0	0	0		+	+	
Iso-amyl alcohol	1.3	70.0 (±0.0)	67.0 (±0.1)		+	++	
Ethyl acetate	0.68	95.4 (±0.1)	96.6 (±0.2)	97.2 (±0.3)	++	+++	++++

[a] Values denote % palmitoyl rutin ester extracted into the organic phase, expressed as the average of duplicate measurements (±SD)

[b] Symbols (+, ++, +++) denote levels of rutin present in the water phase

Table 2 Bioconversion and extraction yield of high purity fatty acid esters of rutin following lipase-catalyzed production

Reaction system	Bioconversion (%)[a]	Purity (%)[b]	Extraction yield (%)[a]
Rutin/palmitic Acid	73–77	>97	74–90
Rutin/lauric Acid	70–72	>98	80–81

Batch reactions were catalyzed by Novozym 435 lipase, employing excess fatty acid substrate (1:4 ratio), dried acetone and 3Å molecular sieves at 50 °C and 200 rpm until equilibrium was achieved (96–144 h)

[a] Results were expressed as a range following multiple (i.e., 4 for R/PA, 2 for R/LA) trials using 10–30 mM rutin substrate

[b] Purity of the isolates was reported as the minimum purity obtained following 4 (R/PA) or 2 (R/LA) trials, respectively

separation. This selective extraction phenomenon was presumed to be related to lower water solubility of the rutin esters following the incorporation of acyl side chains, as well as subsequent differences in partitioning behavior of the compounds between the extraction phases. Bioconversion, purity and extraction yield were summarized in Table 2. Overall, ester purity was consistently high while extraction yield (74–90%) varied based on proper handling during the extraction and drying process. Moreover, rough estimates suggest that up to 2.4 g of pure palmitoyl rutin ester was obtained for each liter of solvent (i.e., acetone, ethyl acetate and heptane) employed. This methodology was also successfully extended to the isolation of lauroyl rutin ester following the biosynthesis reaction of rutin with lauric acid, with results detailed in Table 2. In this case, it was estimated that up to 1.9 g of pure lauroyl rutin ester was obtained for each liter of solvent employed, with some room for improvement (i.e., only ~81% extracted) based on handling techniques during the extraction process.

Characterization of Ester Products

The ability of each solvent to selectively extract either reaction substrates or fatty acid rutin ester products was monitored by HPLC. Figure 1 shows the profile of the palmitoyl rutin ester following biosynthesis and isolation using the optimized two-step extraction process detailed above. Palmitoyl rutin ester (RT 18.1 min) purity was >99% in this instance, residual rutin (RT 8.6 min) was present as well (<1%) and palmitic acid (RT 22.5 min) was not detected in the system. HPLC–ELSD analysis of the lauroyl rutin ester (RT 15.8 min) also revealed the presence of a highly pure (>98%) compound (Fig. 2). Furthermore, ESI–MS characterization was carried out on the fatty acid rutin ester products, confirming that in each case only a single fatty acid was acylated to each molecule of rutin; more specifically, the palmitoyl rutin ester had its major ion peak $[M-1]^-$ at *m/z* 847.38 and the lauroyl rutin ester exhibited its major ion peak $[M-1]^-$ at *m/z* 791.32. Lastly, the acylation position (4‴–OH on the rhamnose moiety) depicted in Figs. 1 and 2 denotes the most likely position for attachment of the fatty acid moiety, given the regioselective nature of the lipase and the nature of the hydroxyl groups on the rutin structure [10, 15, 21].

In conclusion, this work details the biosynthesis of both long and medium-chain fatty acid esters of rutin via lipase-catalyzed esterification, whereby products were isolated in high purity (>97–98%) and reasonable yields (74–90%). This relatively fast, easy and effective method for the extraction of gram quantities of ester product was based on a two-step extraction process, and was ideal for relatively quick processing following scaled-up batch reactions. This

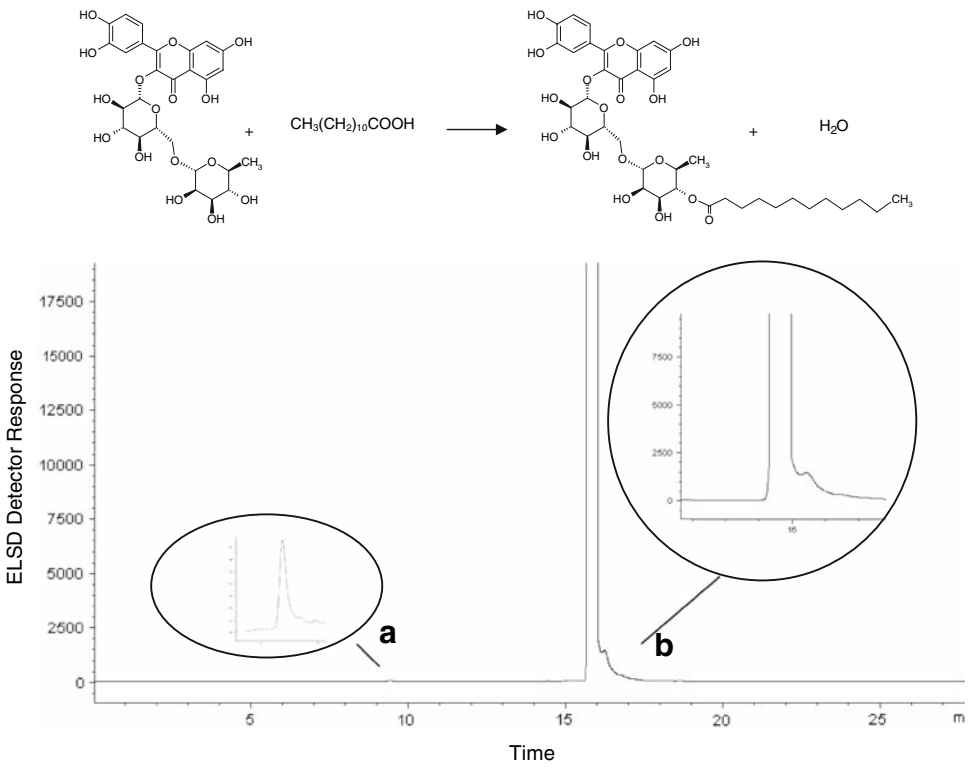

Fig. 2 HPLC–ELSD profile of lauroyl rutin ester product following biosynthesis and isolation using a two-step extraction process; peak *a* identified as residual rutin substrate (<2%) and peak *b* identified as lauroyl rutin ester product (>98%). Note the biosynthesis reaction of rutin with lauric acid (ratio 1:4) was catalyzed by Novozym 435 lipase at 50 °C and 200 rpm in acetone (>96 h). HPLC analysis was carried out on a RP-C8 column, 0.800 mL/min flow, by gradient elution using acidified water and acetonitrile. ELSD settings for evaporator, nebulizer and gas flow corresponded to 90 °C, 50 °C and 1.2, respectively

simple alternative to preparative chromatography has the potential to drastically increase availability of these modified flavonoids, and allow for more widespread investigations aimed at identifying and expanding their uses in a range of interesting areas.

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