

Volatile compounds of Croatian cheese in a lamb skin sack prepared from a mixture of goat's and cow's milk

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Abstract

Volatile aroma compounds are one of the main parameters in consumer choice and acceptance of cheese. The traditional way of making cheese, still preserved in Croatia, is the anaerobic ripening of curds in a lamb or goat sack. Cheeses produced in such a way have a specific aroma. The objective of this study was to determine the volatile compounds of Croatian cheese in a lamb skin sack prepared from a mixture of goat's and cow's milk and relate them with the aroma of this traditional cheese. The cheese extracts were obtained by headspace solid-phase microextraction (HS-SPME) and ultrasonic solvent extraction (USE) and analysed by gas chromatography-mass spectrometry (GC-MS). HS-SPME was performed at different temperatures (40, 50 and 60°C) using 2 fibres, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre and carboxen/polydimethylsiloxane (CAR/PDMS) fibre. Increasing the extraction temperature resulted in higher sorption of fatty acids on both fibres. A total of 28 volatile compounds were identified in the cheese samples including 13 acids, 10 esters, 2 alcohols, 2 hydrocarbons and 1 ketone. Results revealed that fatty acids were the predominant group of volatile compounds in all samples. In samples obtained by HS-SPME, the most abundant were hexanoic, octanoic, butanoic, acetic and decanoic acid while (*Z*)-octadec-9-enoic, hexadecanoic, decanoic and tetradecanoic acid were the most abundant in the sample obtained by USE.

1. Introduction

From ancient times people have used fermentation to preserve perishable food. Cheese is one of the most popular fermented dairy products and is produced in countries throughout the world. There are various processes for making cheese, but the basic method entails culturing milk for various lengths of time. According to a legend, cheese was made accidentally by an Arabian merchant who put milk into a pouch made from a sheep's stomach, as he set out on a day's journey across the desert. The rennet in the lining of the pouch, combined with the heat of the sun, caused the separation of milk into curd and whey. He discovered that whey can quench his thirst, and cheese (curd) can quench his hunger.

This traditional way of making cheese is still preserved in Croatia, Bosnia and Herzegovina, Montenegro, Turkey, Lebanon and eastern Algeria (Kostelac *et al.*, 2020). The main specificity of these cheeses is anaerobic ripening in a sack made of the whole lamb or goat body skin. Sacks have different local

names, such as mišina (Croatia), mijeh (Bosnia and Herzegovina and Montenegro), tulum (Turkey) and chekoua (Algeria) (Tudor Kalit *et al.*, 2020).

The aroma of cheese is a key parameter in consumer choice and acceptance. Freshly made curds of various cheeses have a largely similar flavour and aroma. The complex microbial and biochemical changes that occur during ripening have a significant impact on the formation of cheese flavour and aroma. The cheese flavour is concentrated in the water-soluble fraction (peptides, amino acids, organic acids and amines). In contrast, the aroma is mainly concentrated in the volatile fraction (organic acids, aldehydes, amines, esters) (Mikulec *et al.*, 2010). Cheeses that ripen in animal skin have a specific aroma due to the medium in which they ripen (Vrdoljak *et al.*, 2018).

Production of cheese in a sack has a long tradition in Dalmatia, a Croatian region known for its dry and hot sub-Mediterranean and Mediterranean climate. It dates back to the time of the ancient Illyrians. Their shepherds lived a nomadic lifestyle and stored cheese in sheep skin

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to supply themselves with food throughout the year. The first production of this type of cheese was recorded in the area of mountain Dinara, Dalmatia. Nowadays is mainly produced on small family farms of Dalmatian Zagora (particularly in the Šibenik-Knin area) with their own livestock for milk production. Industrial production from pasteurised milk using mesophilic dairy cultures is present to a lesser extent (Tudor Kalit *et al.*, 2020). The cheese in a sack was originally produced from raw sheep's milk, while today, cow's and goat's milk or their mixtures are used in addition to sheep's milk (Frece *et al.*, 2019).

The technology most similar to the production technology of Croatian cheese in a sack is that of tulum cheese (Turkey) whose aroma and volatile compounds profile have been well-researched (Hayaloglu *et al.*, 2007; Hayaloglu and Karabulut, 2013a; Hayaloglu and Karabulut 2013b; Akpınar *et al.*, 2017; Gursoy *et al.*, 2018; Atik *et al.*, 2021). Kostelac *et al.* (2020) studied the volatiles of Croatian cheese in a sack prepared from sheep's milk during the ripening period. The authors compared the composition of aroma compounds and the sensory properties of the cheese produced with and without the addition of starter cultures. Tudor Kalit *et al.* (2014) studied Croatian cheese in a lamb skin prepared from ovine raw milk to determine compositional, biochemical and sensory changes over 60 days of ripening. To our knowledge, there are no studies about the volatiles of Croatian cheese in a sack prepared from a mixture of cow's and goat's milk.

The aim of this study was to explore the volatile compounds of Croatian cheese in a sack prepared from a mixture of cow's and goat's milk and relate them with a specific aroma of this cheese. In order to obtain a more complete insight into the chemical composition of cheese volatile compounds two methods of isolation were used: headspace solid-phase microextraction, HS-SPME, using two different fibres and performed at three different temperatures (40, 50 and 60°C) and ultrasonic solvent extraction (USE).

2. Materials and methods

2.1 Reagents and cheese sample

The solvents diethyl ether and pentane, purchased from Kemika (Zagreb, HRV), were distilled before usage. Anhydrous Na₂SO₄ was purchased from Fluka Chemie (Buchs, CHE). Cheese in a sack prepared from a mixture of goat's (30%) and cow's milk (70%) was obtained from cheese factory I-Pak, Pakovo Selo, Drniš, Croatia. The cheese was produced from pasteurised milk with the addition of mesophilic dairy cultures and left to ripen for 45 days in lambskin.

2.2 Headspace solid-phase microextraction

Extraction was performed using manual SPME fibres (Supelco Co., Bellefonte, USA) coated with either divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) or carboxen/polydimethylsiloxane (CAR/PDMS). Fibres were conditioned prior to use according to the manufacturer's instructions by inserting them into the GC injector port. For the HS-SPME extraction, 1 g of cheese was placed in a 15-mL amber glass vial and hermetically sealed with a PTFE/silicone septum. The effects of different extraction temperatures (40, 50 and 60°C) were studied. The vial was maintained in a H₂O bath at the same temperature during equilibration (15 mins) and extraction (40 mins) time. After sampling, the SPME fibre was withdrawn from the needle, removed from the vial, and inserted into the injector (250°C) of the GC/MS system for 7 mins, where the extracted volatiles were thermally desorbed directly onto the GC column.

2.3 Ultrasonic solvent extraction

Ultrasonic solvent extraction (USE) was performed in an ultrasound cleaning bath (Transsonic Type 310/H, Germany) by the mode of indirect sonication, at the frequency of 35 kHz at 25±3°C. Forty grams of the cheese samples were dissolved in 40 mL of distilled H₂O in a 250-mL flask. Anhydrous Na₂SO₄ (1.5 g) was added, and the sample was extensively vortexed. For extraction mixture of solvents pentane/diethyl ether 1:2 (v/v) was used. Sonication was held for 30 mins. After sonication, the organic layer was separated by centrifugation and filtered over anhydrous Na₂SO₄. The aqueous layer was returned to the flask and another batch of the same extraction solvent (20 mL) was added and the sample was extracted by ultrasound for 30 mins. The organic layer was separated from the previous one, filtered over anhydrous Na₂SO₄, and the aqueous layer was sonicated a third time for 30 mins with another batch (20 mL) of the extraction solvent. The combined organic extracts were concentrated by fractional distillation and used for GC-MS analyses.

2.4 Gas chromatography-mass spectrometry analysis

The analyses of the volatile compounds were carried out with Agilent Technologies (Palo Alto, CA, USA) GC-MS system, GC model 7890A apparatus equipped with a 5975C mass-selective detector and using a non-polar HP-5MS fused-silica cap. column (5%-phenylmethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 mm, Agilent J&W). The oven temperature was programmed isothermal at 70°C for 2 mins, rising from 70 to 200°C at 3°C/min, and then held isothermal at 200°C for 15 mins; injector temperature, 250°C; detector

temperature, 300°C; carrier gas, He (1.0 mL/min); injection volume, 1 µL; split ratio, 1:50. MS conditions were: ionization voltage 70 eV, ion source temperature 280°C, mass range 30–350 mass units.

The individual peaks were identified by comparison of their *RI* (determined rel. to the t_R of *n*-alkanes (C₉–C₂₅) for the HP-5MS column) with those of authentic samples and literature values, as well as by comparing their mass spectra with the Wiley 275 MS library and NIST17 mass spectral database. The percentage composition of the samples was computed from the GC peak areas using the normalization method (without correction factors).

3. Results and discussion

3.1 Headspace volatiles

Headspace solid-phase microextraction was performed at different temperatures (40, 50 and 60°C) using 2 fibres, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre and carboxen/polydimethylsiloxane (CAR/PDMS) fibre. According to the results of the volatile compounds analysis, a total of 21 different volatile compounds including 8 carboxylic acids, 9 esters, 2 alcohols, 1 ketone and 1 hydrocarbon were detected (Table 1).

Some researchers (Bezerra *et al.*, 2016; Sýkora *et al.*, 2020) reported that increasing extraction temperature leads to the release of a greater quantity of volatile compounds into the headspace since the rise in the temperature is proportional to the increase in the analyte diffusion coefficient. Still, high temperatures may also provoke the degradation of some compounds. In the case of cheese, a high extraction temperature could result in their melting, which may affect its physical properties in terms of diffusion of analytes into the headspace. In this investigation, no significant differences in results between headspace solid-phase microextractions performed at 50 and 60°C were observed. However, the least identified compounds were at 40°C (18), while at higher temperatures (50 and 60°C) 21 and 20 compounds were identified, respectively. Also, an increase in extraction temperature resulted in higher sorption of carboxylic acids on both fibres (DVB/CAR/PDMS: 44.80% (40°C), 72.50% (50°C), 82.40% (60°C); CAR/PDMS: 40.50% (40°C), 63.20% (50°C), 79.70% (60°C)).

In this study, carboxylic acids have been the predominant group of volatile compounds. It is not uncommon because acids are important and dominant compounds in many kinds of cheeses. Most of the acids detected in cheeses generally occur because of lipolysis of milk fat or from the fermentation of lactose or lactic

acid. Fatty acids with long-chain of carbon atoms (C14–C18:3) can be formed by lipolysis of triglycerides, those with medium-chain of carbon atoms (C10–C12) can be formed by degradation of lactose and amino acid, and short-chain fatty acids can also be formed by the oxidation of aldehydes, ketones and esters (Molimard and Spinnler, 1996). Free fatty acids, especially short- and medium-chain fatty acids, directly contribute to the cheese flavour (Park, 2001). In addition, carboxylic acids are not only aroma components as such, but can also be precursors for new aroma compounds, like methyl ketones, alcohols, lactones, aldehydes and esters (Collins *et al.*, 2003).

The most abundant carboxylic acids identified in this research were hexanoic, octanoic, butanoic, acetic and decanoic acids. Hexanoic, octanoic and decanoic acid, also known as caproic, caprylic and capric acid, are commonly associated with the distinctive odour of goat dairy products. The smell of these acids is described as goat-like, waxy and cheesy (Akpınar *et al.*, 2017). Butanoic acid has been reported to give a rancid-cheese-like aroma (Demirci *et al.*, 2021). This acid is produced mainly by lipolysis, through catabolism of some amino acids (such as norvaline, threonine, glutamine acid and methionine) or butyric fermentation of lactose (Bosset *et al.*, 1993). Acetic acid gives the cheese a pungent and vinegar aroma (Vrdoljak *et al.*, 2018). In addition to the fermentation of lactose, this acid is also formed from the oxidation of lactate by the action of non-starter lactic acid bacteria (NSLAB) or from the catabolism of the amino acids alanine and serine by starter bacteria or NSLAB (Rehman *et al.*, 2000). 3-Methylbutanoic (isovaleric) and 2-methylbutanoic acid, both found in lower concentrations, originated from isoleucine and leucine metabolism. Those branched-chain fatty acids are associated with the sweaty and rancid aroma of cheese (Atik *et al.*, 2021).

Hexanoic, octanoic and decanoic acids were the most abundant fatty acids present in Izmir tulum cheese samples produced from cow's milk and Izmir tulum cheese samples produced from a mixture of ewe's, goat's and cow's milk (Akpınar *et al.*, 2017). Butanoic, acetic and caproic acids were reported as the most present acids, both in tulum cheese that ripens in animal skin and in other Turkish cheeses (Hayaloglu *et al.*, 2007; Hayaloglu and Karabulut, 2013a). These authors also identified caprylic acid, 3-methylbutanoic and 2-methylbutanoic acid. They found that short-chain fatty acids are more present in tulum cheeses than medium-chain acids and reported significant differences in the concentrations of 2-methylbutanoic and 3-methylbutanoic acid depending on the ripening medium (Hayaloglu *et al.*, 2007). Short- and medium-chain fatty

Table 1. Volatiles of cheese in a sack isolated by HS-SPME and USE.

No.	Compound name	RI HP-5MS	Peak area (%)			USE
			HS-SPME I/II			
			40°C	50°C	60°C	
1	ethanol	< 900	3.5/2.2	1.9/2.9	0.4/0.7	-
2	acetic acid	< 900	2.2/8.0	9.4/16.1	11.6/15.4	-
3	butan-2-ol	< 900	16.3/9.2	2.9/-	3.0/-	-
4	butan-2-one	< 900	4.4/25.6	1.3/15.1	2.1/5.0	-
5	propanoic acid	< 900	-/-	-/0.5	-/0.5	-
6	butanoic acid	< 900	9.6/15.3	8.5/18.5	8.3/19.3	-
7	ethyl butanoate	< 900	1.8/2.2	-/1.0	-/0.7	-
8	3-methylbutanoic acid	< 900	3.1/2.0	2.2/2.5	1.5/2.1	1
9	2-methylbutanoic acid	< 900	1.8/1.1	0.6/1.0	0.9/1.2	0.6
10	1-methylpropyl butanoate	945	2.8/1.7	0.2/1.3	-/0.8	-
11	m-xylene	945	-/-	-/-	-/-	0.3
12	hexanoic acid	991	22.8/12.1	33.9/20.3	22.4/31.1	7.6
13	2,2,4,6,6-pentamethylheptane	1000	3.9/2.4	-/1.9		-
14	ethyl hexanoate	1008	5.5/2.5	4.2/2.6	1.0/3.9	-
15	propyl hexanoate	1104	1.2/tr.	1.2/tr.	0.4/0.7	-
16	2-methylpropyl hexanoate	1144	6.9/3.0	7.6/3.9	3.0/4.1	-
17	octanoic acid	1189	5.3/2.0	15.4/4.3	25.2/9.1	9.6
18	ethyl octanoate	1207	2.4/tr.	3.7/1.5	2.6/2.0	-
19	2-phenylacetic acid	1270	-/-	-/-	-/-	0.4
20	propyl octanoate	1286	-/-	tr./tr.	0.6/tr.	-
21	2-butyl octanoate	1340	-/-	1.4/tr.	1.5/-	-
22	decanoic acid	1381	-/tr.	2.5/tr.	12.5/1.0	16.7
23	ethyl decanoate	1406	-/tr.	0.9/tr.	1.3/tr.	-
24	dodecanoic acid	1590	-/-	-/-	-/-	7.2
25	δ -dodecalactone	1719	-/-	-/-	-/-	0.2
26	tetradecanoic acid	1796	-/-	-/-	-/-	11.2
27	hexadecanoic acid	1995	-/-	-/-	-/-	19.2
28	(Z)-octadec-9-enoic acid	2176	-/-	-/-	-/-	23.9
Total identified (%)			93.5/89.3	97.8/93.4	98.3/97.6	97.9

RI: Retention index determined relative to a homologous series of *n*-alkanes (C₉ - C₂₅) on a HP-5MS column, I: HS-SPME with DVB/CAR/PDMS fibre, II: HS-SPME with CAR/PDMS fibre, USE (solvent *n*-pentane: Et₂O 1: 2, v/v), - = not detected, tr. = traces < 0.1%.

acids in four Söğle cheese samples were listed as capric, caprylic, caproic and butanoic acid according to their contents (Gursoy *et al.*, 2018). Acetic acid, butane-2,3-diol, ethyl acetate and α -pinene were found as the dominant volatiles in the samples of tulum cheese produced in Anamur from raw goats' milk and ripened in goat skin (Atik *et al.*, 2021). Kostelac *et al.* (2020) investigated the volatiles of Croatian cheese in a sack prepared of sheep's milk with and without the addition of starter culture during the ripening period. They found butanoic, acetic, caproic, caprylic, 3-methylbutanoic and 2-methylbutanoic acid. Compared to our study, in which carboxylic acids were the most abundant compounds, the concentration of acids was much lower.

Esters are common cheese volatiles. Most of them are usually described as having sweet, fruity (especially

ethyl esters), floral notes in low concentrations and yeast notes in high concentrations. Due to their high volatility at room temperature, they directly contribute to the aroma of cheese (Gursoy *et al.*, 2018). Furthermore, these compounds can contribute to the aroma of cheese by minimizing the bitterness of amines and the sharpness of fatty acids (Curioni and Bosset, 2002). The biosynthesis of esters proceeds through two enzymatic mechanisms: esterification of alcohols and carboxylic acids and through alcoholysis of alcohols and acylglycerols or alcohols and fatty acyl-CoAs derived from the metabolism of fatty acids, amino acids and/or carbohydrates (Liu *et al.*, 2004).

Nine esters were detected in this investigation. 2-Methylpropyl hexanoate (isobutyl caproate), ethyl hexanoate and ethyl octanoate were the main esters

identified in cheese while 1-methylpropyl butanoate, ethyl butanoate, ethyl decanoate, propyl hexanoate and propyl octanoate were found in lower concentrations. According to Liu *et al.* (2004), ethyl butanoate contributes to the formation of apple, banana, sweet and fruity notes, ethyl hexanoate notes of banana, pineapple and wine, ethyl octanoate notes of pear, pineapple, apricot and flower and ethyl decanoate notes of apple, brandy, grape, fruity and oily notes. 2-Methylpropyl hexanoate was also found in Canak cheese, four Söğle cheeses and tulum cheeses ripened in plastic barrels (Hayaloglu and Karabulut, 2013a; Gursoy *et al.*, 2018; Tekin and Guler, 2021).

Many authors reported ethyl esters as the most common esters in cheese. Ethyl hexanoate, ethyl octanoate, ethyl decanoate and acetyl acetate were found in the volatile fraction of Izmir Tulum cheeses (Akpınar *et al.*, 2017). Hayaloglu *et al.* (2007) reported that a ripening medium (goat skin or plastic barrel) can significantly contribute to the share of ethyl butanoate, ethyl caproate, 3-methylbutyl ethanoate and propyl ethanoate. Ethyl hexanoate, ethyl butanoate, methyl butanoate and ethyl acetate were found at the highest levels in 11 different varieties of Turkish cheese (Hayaloglu and Karabulut, 2013a). The most abundant esters, present in Söğle cheese samples, were ethyl decanoate, ethyl octanoate and ethyl hexanoate (Gursoy *et al.*, 2018). Among seven esters found in Croatian cheese in a sack prepared from sheep's milk, ethyl esters: ethyl acetate, ethyl butanoate, ethyl hexanoate and ethyl octanoate were dominant (Kostelac *et al.*, 2020).

Alcohols are considered important volatile compounds that can give the cheese an alcoholic, winey, sweet, fruity and pungent note (Hayaloglu and Karabulut, 2013a). Some authors reported that the pasteurization of cheese milk has a negative effect on the formation of alcohol in cheese (Ortigosa *et al.*, 2005). On the other hand, Rehman *et al.* (2000) suggested that the pasteurization process does not significantly influence the formation of alcohols. However, in this research, only two alcohols were detected, ethanol and butan-2-ol. Ethanol, which plays a significant role in the formation of esters, is mostly produced by the fermentation of lactose and by the catabolism of alanine while secondary alcohol butan-2-ol stems mainly from citrate metabolism (Gursoy *et al.*, 2018; Tekin and Guler, 2021). These two alcohols are common in many kinds of cheeses. Ethanol was found in high concentrations in all samples of eleven varieties of Turkish cheese, except in Civil cheese (Hayaloglu and Karabulut, 2013a). Among the 15 alcohols identified in tulum cheeses, ethanol, butan-2-ol and 3-methylbutanol were the most abundant alcohols during ripening (Tekin and Guler, 2021). Ethanol was

also the most common alcohol in cheese samples of Croatian cheese in a sack prepared of sheep's milk. Besides ethanol and butan-2-ol (which was found only in one sample), 6 more alcohols were identified: 2-methylpropan-1-ol, 1-butoxypropan-2-ol, 3-methylbutan-1-ol, 2-methylbutan-1-ol, hexan-1-ol and pentan-2-ol (Kostelac *et al.*, 2020).

Only one ketone, butan-2-one, was detected in this investigation. Ketones are formed by the enzymatic oxidation of fatty acids to β -keto acids and then their decarboxylation to methyl ketones. Methyl ketones are the main ingredients contributing to the aroma of mouldy cheeses (McSweeney and Sousa, 2000). Due to their unique aroma and low perception threshold, they can significantly contribute to the aroma of the cheese (Vrdoljak *et al.*, 2018). Butan-2-one, which is responsible for the buttery and sour milk aroma in many kinds of cheeses, is derived from butane-2,3-dione (diacetyl) during lactose or citrate metabolism by microorganisms (Gursoy *et al.*, 2018; McSweeney and Sousa, 2000).

Many authors reported the presence of butan-2-one in cheeses that ripen in animal skin. Demirci *et al.* (2021) reported that butan-2-one was the most abundant ketone at the end of ripening. Similarly, Hayaloglu *et al.* (2007) reported that the highest methyl ketone detected in tulum cheeses was butan-2-one.

One of the most common hydrocarbons found in cheeses that ripen in animal skin, 2,2,4,6,6-pentamethylheptane (Vrdoljak *et al.*, 2018), is the only hydrocarbon detected in our study. Most hydrocarbons in cheese do not contribute to aroma due to their high perception threshold values (Tekin and Guler, 2021).

3.2 Volatile and semi-volatile compounds from ultrasonic solvent extraction extract

Twelve compounds were isolated by USE. Except for *m*-xylene and δ -dodecalactone, identified in low concentrations (< 0.3%), all other compounds were fatty acids. The most abundant were (*Z*)-octadec-9-enoic acid (oleic), hexadecanoic acid (palmitic), decanoic acid (capric) and tetradecanoic acid (myristic) which together accounted for 70.95% of the total sample. Additionally, dodecanoic and 2-phenylacetic acid, which were not identified by HS-SPME, were detected.

Similar results were reported by Yilmaz *et al.* (2005) who found that palmitic, oleic and myristic acids were at the highest level in tulum cheese, while Serhan *et al.* (2010) reported that palmitic, oleic and capric acid was the predominant free fatty acids in traditional Darfiyeh cheese made from raw goat's milk. Tudor Kalit *et al.* (2014) reported that palmitic, oleic and stearic acids

were the predominant free fatty acids in Croatian cheese in a lamb skin sack prepared from raw ovine milk. Despite the higher proportion of long-chain fatty acids, it is considered that, due to their high perception threshold values, they do not have a large impact on the aroma of cheese (Molimard and Spinnler, 1996).

Commonly the main step in GC analysis of fatty acids (especially-long chain ones) is their derivatization into suitable volatile esters (methyl-, ethyl- and isopropyl -) (Dolowy and Pyka, 2015). In this study, USE in combination with GC-MS analysis is proved to be a simple and fast method for the isolation and identification of long-chain fatty acids without their prior derivatization.

4. Conclusion

This study provided the first report on the volatile composition of Croatian mixed cow and goat cheese ripened in a lambskin sack. The aim of the research was to define the volatile aroma profile of this traditional cheese, in order to contribute to its standardization and protection. Regardless of the isolation method, HS-SPME or USE, fatty acids were the dominant compounds in all cheese samples. In addition, the different temperatures applied in the HS-SPME method affected the fatty acid content. Increasing the extraction temperature resulted in higher sorption of fatty acids on both fibres used.

Conflict of interest

The authors declare no conflict of interest.

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