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Chain separation of monounsaturated fatty acid methyl esters by argentation thin-layer chromatography

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Abstract

A technique for separating methyl esters of monounsaturated fatty acids by argentation chromatography using silver nitrate-impregnated TLC plates is described. Monounsaturated fatty acid methyl esters are separated from polyunsaturated and saturated fatty acid methyl esters and the monounsaturated fatty methyl esters are resolved according to chain length. *cis* isomers are well resolved from the corresponding *trans* isomers. R_F values for individual monounsaturated fatty acids are very reproducible. The potential of the technique in metabolic studies is demonstrated in the chain elongation of [^{14}C]-18:1($n-9$) and delta-9 desaturation of [^{14}C]-18:0 by human skin fibroblasts. Recoveries of individual [^{14}C]-fatty acids for scintillation counting exceed 94%.

Keywords: Argentation; Fatty acid methyl esters; Monounsaturated fatty acids; Oleic acid; Nervonic acid

1. Introduction

Fatty acid methyl esters (FAMEs) can be separated and quantified by gas chromatography (GC) [1] and high-performance liquid chromatography (HPLC) [2], and these techniques have been applied to study fatty acid metabolism using radiolabelled compounds [3–7]. Radiolabelled FAMEs separated by these techniques can be counted directly with radioactivity detectors or fractions collected for analysis by liquid scintillation counting [3–7]. Argentation thin-layer chromatography (TLC) has also been used routinely to study the metabolism of polyunsaturated fatty acids (PUFAs) using radioisotopes [8–16]. Although radio–gas chromatography has been used to study the metabolism of the monoene erucic acid, 22:1$n-9$ [5] there are no silver nitrate TLC techniques for studying the metabolism of radiolabelled monounsaturated fatty acids (MUFAs). In this paper we report a simple silver nitrate technique that resolves monoenes according to chain length and separates them from other classes of FAME. The use of the technique in metabolic studies is demonstrated in the chain elongation of [^{14}C]-18:1($n-9$) and delta-9 desaturation of [^{14}C]-18:0 by human skin fibroblasts.

2. Materials and methods

2.1. Materials

Butylated hydroxytoluene (BHT) and standard

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FAMEs (at least 99% pure) were obtained from Sigma (Poole, UK). Silver nitrate was obtained from Johnson Matthey (Royston, UK). [1-^{14}C]-18:1*n*−9 (54.7 mCi/mmol), [1-^{14}C]-18:0 (53.9 mCi/mmol), [1-^{14}C]-*n*-hexadecane (50–60 mCi/mmol) and radioactive ink were purchased from Amersham International (Aylesbury, UK). Ecoscint A scintillation fluid was supplied by National Diagnostics (NJ, USA). Konica A2 X-ray film was supplied by MAS (Stirling, Crief, UK). Silica gel 60 TLC plates (Merck 5721, 20 cm×20 cm×0.25 mm) were purchased from BDH (Poole, UK). CP-Wax 51 (25 m×0.53 mm I.D.) and CP Sil 5 (25 m×0.53 mm I.D.) columns were obtained from Chrompack (The Netherlands). All solvents were of HPLC grade and were purchased from Rathburn (Walkerburn, UK).

2.2. Preparation of [^{14}C]-fatty acid methyl esters

To generate intermediates of metabolism, cultured human skin fibroblasts were incubated with either 0.5 μCi [1-^{14}C]-18:1*n*−9 or 0.5 μCi [1-^{14}C]-18:0 for 24 h as described previously [17]. Total lipid was isolated and FAMEs prepared by transmethylation as described previously [17,18]. In these experiments [1-^{14}C]-*n*-hexadecane was added as internal standard. FAMEs were dissolved in hexane containing 0.01% BHT.

2.3. Separation of MUFA mixtures by argentation TLC

Silica gel 60 TLC plates (20 cm×20 cm×0.25 mm) were sprayed uniformly with 20 ml acetonitrile containing 2 g silver nitrate until the plates were saturated [16]. The plates were air-dried in subdued light, heated at 110°C for 30 min to achieve activation and used within 1 h [16]. FAME mixtures were applied to the silver nitrate impregnated TLC plates as a narrow band over 1–5 cm using a Hamilton micro-syringe at 0.5–1.0 mg/cm. Approximately 100 μg/cm of each component was present in the mixtures using this loading. In other experiments radiolabelled-FAMEs were applied to plates as a narrow band over 2–5 cm at 50 000 dpm/cm. The plates were developed with toluene–hexane (40:60, v/v) to 1 cm from the top in a standard TLC tank. The tank was lined with tissue to saturate the atmosphere with solvent. Separated FAMEs were visualised by charring, autoradiography or under UV light after spraying with dichlorofluorescein [16,18].

2.4. Quantification of [^{14}C]-FAMEs by argentation TLC

After chromatography, plates were marked with radioactive ink (including solvent front and origin) and subjected to autoradiography for 4 days using Konica A2 X-ray film. Areas of silica corresponding to radioactive FAMEs were located, scraped from the plates into vials and 5 ml Ecoscint A added. Radioactivity was determined using a Packard Tricarb 2000CA liquid scintillation analyser [11,18]. In other experiments FAMEs were located, scraped from the plates and eluted using 5 ml ice-cold chloroform–methanol (2:1, v/v) containing 0.01% BHT; 1.25 ml 0.88% KCl was added and the solutions mixed [18]. After separation the chloroform layer was removed in to a clean tube and the solvent removed under nitrogen. FAMEs were redissolved in hexane containing 0.01% BHT and washed with 20% NaCl to precipitate any remaining silver. The hexane layer was removed and an aliquot was counted for radioactivity and the remainder analysed by radio–gas chromatography as described below. Recoveries of individual radioactive methyl esters were at least 93–95% as determined by liquid scintillation counting of individual zones eluted from the plates.

2.5. Identification and quantification of [^{14}C]-FAMEs by radio–gas chromatography

Samples were analysed using a Chrompack CP9000 gas chromatograph using either a CP Wax 51 column (25 m×0.53 mm I.D.) or CP Sil 5 column (25 m×0.53 mm I.D.) with argon–10% carbon dioxide as carrier gas at a flow-rate of 10 ml/min. These wide bore columns permitted high sample loading that was required for the detection of components with low levels of radioactivity. The temperature programme was from 60°C to 220°C at 25°C/min and from 220°C to 250°C at 2°C/min. The instrument was equipped with a stream splitter situated before the flame ionisation detection (FID) system and 90% of the carrier gas was directed to a

combustion oven situated within the GC oven. The resultant gases were measured for radioactivity in a Lab Logic β-ram radio-detector using argon containing 10% methane as a counting gas at a flow of 20 ml/min. [^{14}C]-FAMEs, which had been eluted from thin-layer plates as described above, were added to authentic non-radioactive standards. Separated components were identified by reference to standards and quantified using FID and a recording integrator. Location of radioactivity with authentic standards confirmed the identity of [^{14}C]-FAMEs.

3. Results

Representative lanes of TLC plates developed in toluene–hexane (40:60, v/v) and charred are shown in Fig. 1. Mixtures of monounsaturated methyl esters are resolved according to the chain length; with 18:1$n-9$ clearly separated from 20:1$n-9$ which is separated from 22:1$n-9$ which is separated from 24:1$n-9$. All monounsaturated methyl esters were separated from polyunsaturated and saturated methyl esters (R_F=0.78); although saturates under these conditions did not char. Dienes but not trienes were also separated according to chain length and PUFAs with four or more double bonds co-migrated. The technique is reproducible with R_F values for 18:1$n-9$, R_F=0.15; 20:1$n-9$, R_F=0.21; 22:1$n-9$, R_F=0.27; 24:1$n-9$, R_F=0.33 being recorded over four separate analyses of the same mixture.

The analysis by argentation TLC of the products from the metabolism of [1-^{14}C]-18:1$n-9$ in human skin fibroblasts is shown in Fig. 2. This chromatogram demonstrates that in these cells oleic acid (18:1$n-9$) is metabolised to nervonic acid (24:1$n-9$) with intermediates 20:1$n-9$ and 22:1$n-9$ also being detected. Radio–gas chromatography did not detect these metabolic intermediates except when pooled samples were analysed thereby increasing the amount of radioactivity injected (Fig. 3). Radio–gas chromatography therefore confirmed that skin fibroblasts synthesised nervonic acid with intermediates 20:1$n-9$ and 22:1$n-9$. There was also a number of minor products identified as 18:2$n-9$, 20:2$n-9$ and 20:3$n-9$; these being derived from the desaturation and elongation of [1-^{14}C]-18:1$n-9$ (not shown).

The analysis by argentation TLC of the products of metabolism of [1-^{14}C]-18:0 is shown in Fig. 2. In these cell [1-^{14}C]-18:0 was readily converted to [1-^{14}C]-18:1$n-9$. However, although analysis of pooled samples by radio–gas chromatography using a CP Sil 5 column confirmed the conversion of [1-^{14}C]-18:0 to [1-^{14}C]-18:1$n-9$ there was incomplete separation between substrate and metabolite (Fig. 3). In these experiments the incomplete separation of these two compounds was attributed to the high sample loading and was much poorer when using a CP Wax 52 column (not shown).

4. Discussion

Argentation TLC has successfully been used to separate fatty acid esters according to the number and configuration of double bonds [19]. Various geometric isomers of *cis*- and *trans*-monoenes, dienes or trienes; which are found in heat stressed oils or partially hydrogenated fats, have been isolated by argentation TLC using a number of different solvent systems with subsequent analysis by GC [19]. PUFAs with up to six double bonds can be separated by argentation TLC not only according to the degree of unsaturation but chain length also, but with little or no resolution between $n-6$ and $n-3$ PUFAs [16,19]. It is generally more difficult to separate positional isomers of fatty acids although recently it was reported that positional isomers of C_{18} or C_{20} MUFAs could be resolved by argentation TLC as the phenacyl ester [20]. We have found toluene modified using either acetonitrile or hexane effective in achieving a number of different separations. However, the solvent system used in argentation TLC depends on the separation required [19] and resolution of complex mixtures of fatty acid esters with all possible combinations geometric and positional isomers and double bond configuration may be impossible using a single solvent system.

Argentation TLC is now routinely used to study the metabolism of radioactive PUFAs in a number of different cultured cell lines [11–15]. Although there are many argentation TLC techniques for the separation of PUFAs [11–16,19] there are none that can be applied to study MUFA metabolism. In the present study we describe a solvent system for argentation TLC that resolves monoenes according to

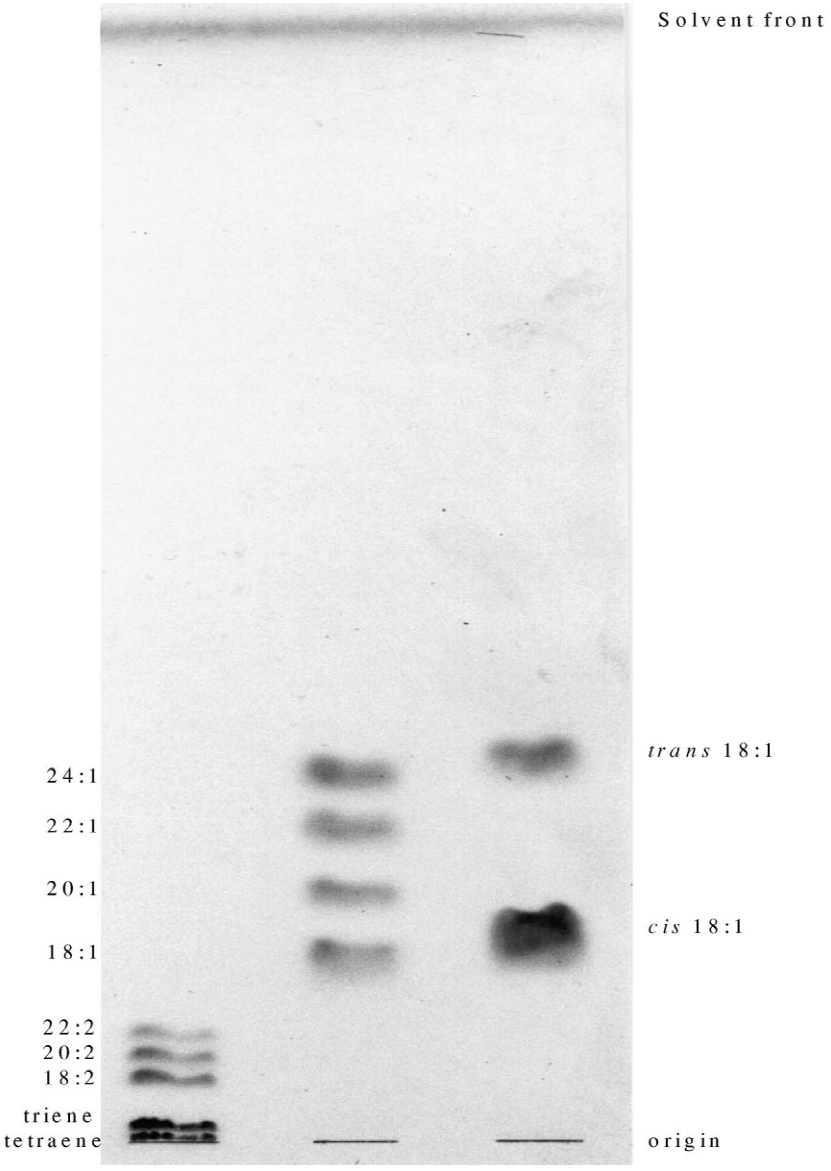

Fig. 1. Chain separation of monounsaturated fatty acid by argentation thin-layer chromatography. FAMEs were analysed by argentation chromatography and visualised by charring as described in Materials and Methods. Mixtures of monounsaturated methyl esters are resolved according to chain length and all are clearly resolved from each other. Monounsaturated methyl esters were separated from polyunsaturated and saturated methyl esters; which under these conditions saturated methyl esters did not char. The corresponding *cis*- and *trans*-ω9 monounsaturated isomers are well resolved.

chain length and separates them from other FAMEs. In metabolic studies human skin fibroblasts metabolised [1-^{14}C]-oleic acid to nervonic acid (24:1$n-9$) through 20:1$n-9$ and 22:1$n-9$ as intermediates. In this study, 18:2$n-9$, 20:2$n-9$ and 20:3$n-9$, metabolic products associated with essential fatty acid deficiency, were also detected using argentation TLC. Human skin fibroblast when incubated with high levels of monoenes manifest signs of essential fatty acid deficiency [17] and this was confirmed in

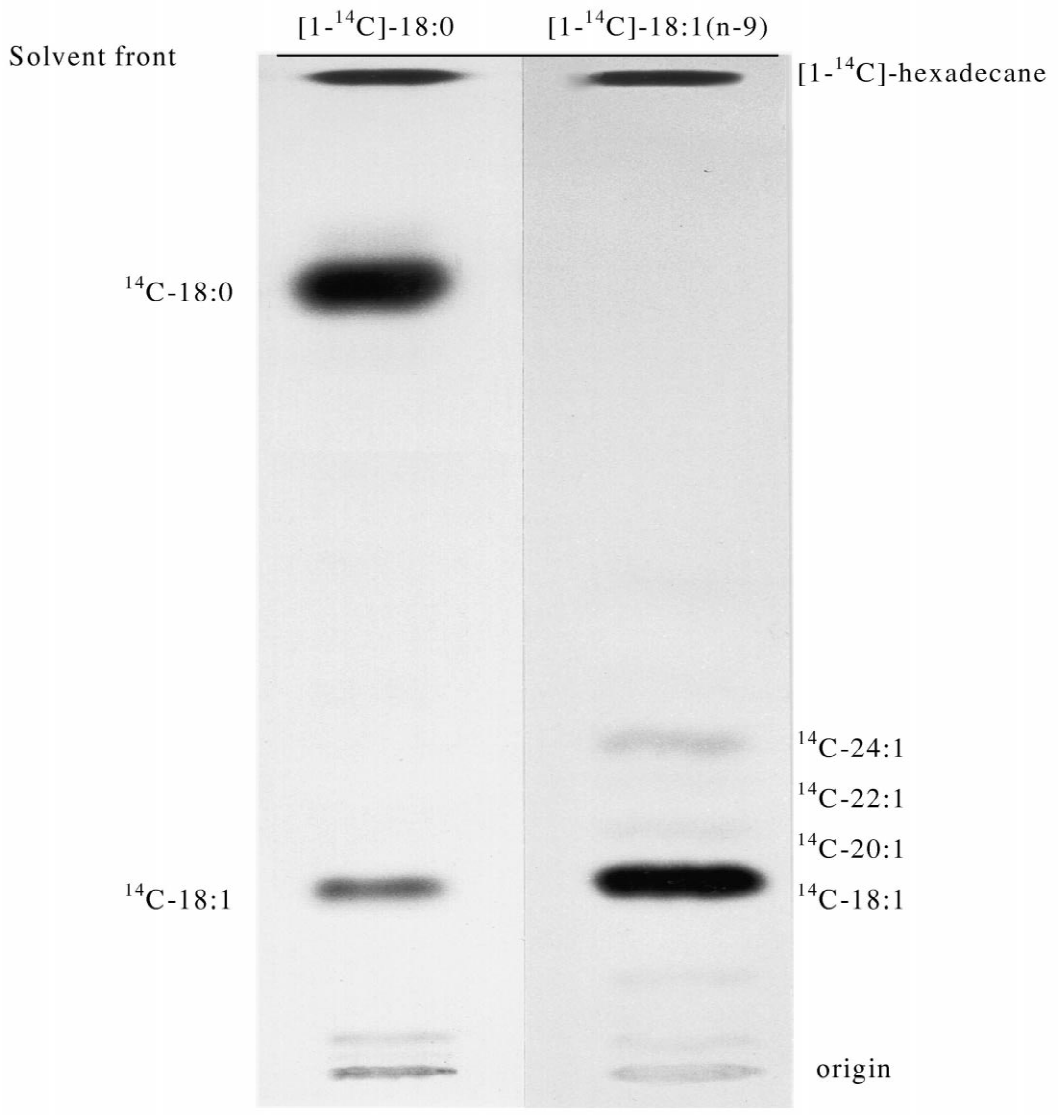

Fig. 2. Argentation TLC analysis of the products from the metabolism of [1-^{14}C]-18:0 and [1-^{14}C]-18:1$n-9$ by cultured skin fibroblasts. Mixtures of [^{14}C]-FAMEs were analysed by argentation chromatography and detected by autoradiography as described in Materials and Methods. The metabolism of [1-^{14}C]-18:0 by delta-9 desaturase to [^{14}C]-18:1$n-9$ is shown as is the elongation of [^{14}C]-18:1$n-9$ to [^{14}C]-24:1$n-9$ with intermediates [^{14}C]-20:1$n-9$ and [^{14}C]-22:1$n-9$ detected.

the present study. It therefore important to note that other metabolic reactions may occur when cells are incubated with either high level of substrate or a deficiency of competing substrate.

Studies of fatty acid metabolism using GC or HPLC equipped with radio-detectors and computer data acquisition for analysis [3–7] have various drawbacks not least the expense of the equipment required [1,2]. It is also known that radio–gas chromatographic analysis requires high specific activities for analysis when flow detectors are used [1,2]. In comparison to argentation TLC, radio–gas chromatography, demonstrated these intermediates of metabolism only when concentrated samples were analysed. Radio–gas chromatography has been used to study the metabolism of [14-^{14}C]-erucic acid in

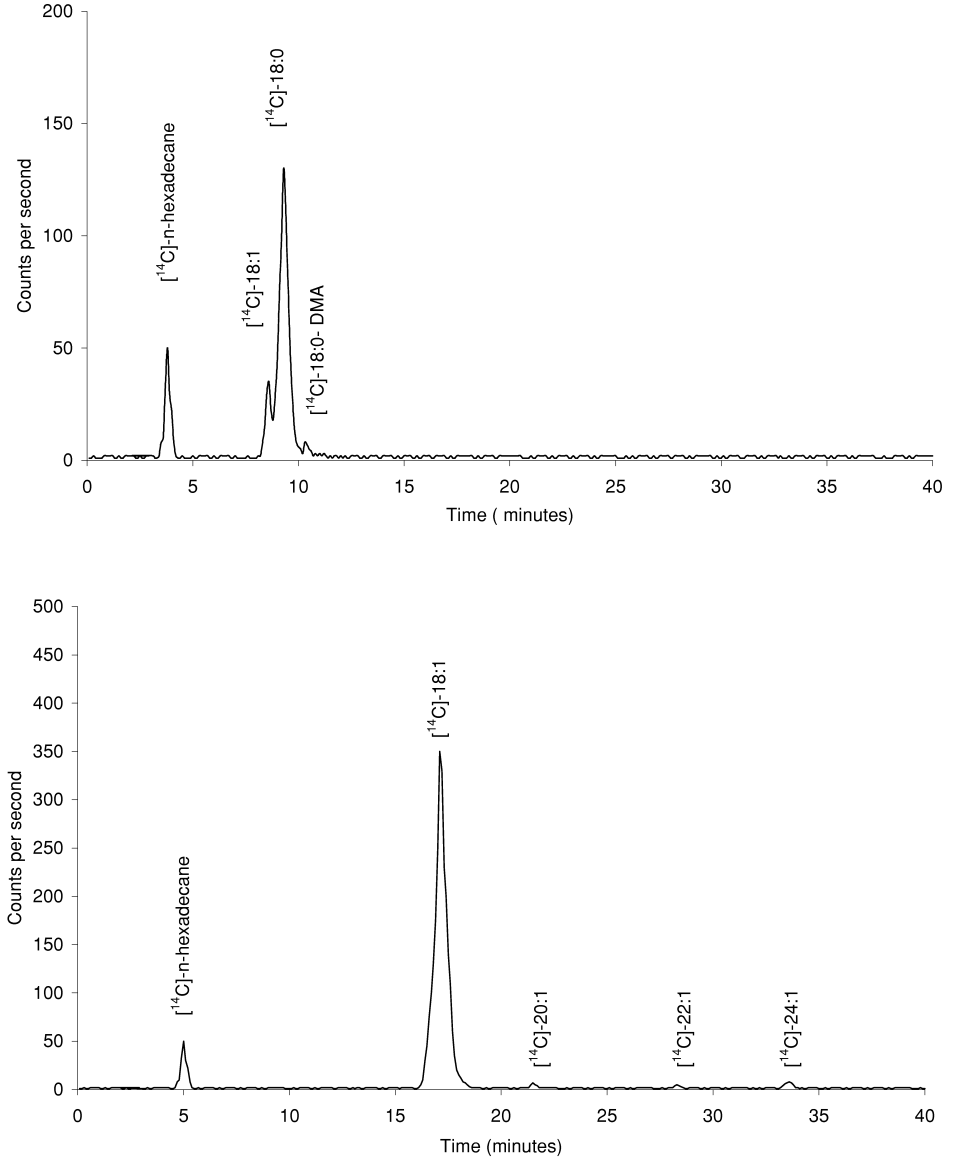

Fig. 3. Radio–gas chromatography analysis of the products from the metabolism of [1-^{14}C]-18:0 or [1-^{14}C]-18:1$n-9$ by cultured skin fibroblasts. [^{14}C]-FAMEs were analysed by radio–gas chromatography as described in Materials and Methods using pooled samples to increase the amount of radioactivity injected. The analysis demonstrated that [1-^{14}C]-18:0 is readily converted to [1-^{14}C]-18:1$n-9$ and [1-^{14}C]-18:1$n-9$ is metabolised to 24:1$n-9$ through 20:1 and 22:1 as intermediates and confirmed the products identified by argentation TLC.

skin fibroblasts [5]. In that study skin fibroblasts were incubated with 4 μCi of [14-^{14}C]-erucic acid [5] eight times more radioactivity as used in the present study further indicating the requirement for high levels of radioactivity when using such detectors. Furthermore, it should be noted that in the present study chromatographic resolution between [^{14}C]-oleic and [^{14}C]-stearic methyl esters was compromised due to the high column loading required to detect this metabolism.

In conclusion the paper describes the use of a simple silver nitrate TLC technique to separate monounsaturated FAMEs according to chain length. The technique can be applied to study the elongation of [1-^{14}C]-oleic acid or desaturation of [1-^{14}C]-stearic acid in cells in culture. In comparison to radio–gas chromatography, the argentation TLC technique is simple, inexpensive, can be operated by relatively unskilled staff and more importantly low levels of radioactivity can be analysed. The technique therefore offers an excellent alternative and complementary method to GC and HPLC for studying elongation of monounsaturated and/or desaturation of saturated fatty acids using radiolabelled substrates.

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