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Short communication

Automated determination of fatty acid methyl ester and *cis*/*trans* methyl ester composition of fats and oils

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Abstract

The determination of the fatty acid composition (as methyl esters, FAMEs) of fats and oils and their *cis*/*trans* (CTME) distribution requires a simple, but manual and time-consuming sample preparation. The so-called BF_3 method is often the preferred procedure. Because FAME/CTME analyses are encountered very frequently in the food industry, an automated, robot-based alternative is proposed which uses the sodium methylate procedure. After sample weighing and the (manual) addition of heptane (2 min), a XYZ robotic autosampler is used for all remaining work, which includes reagent addition, agitation, sample settling and the final injection into the gas chromatograph (10 min). The performance of the sodium methylate and BF_3 methods are compared by analysing some 30 oil and fat samples. The novel procedure is much faster (less than 15 min versus ca. 1 h) and manual sample handling is drastically decreased. The experimental results obtained with the two methods frequently are the same, while small differences can be explained by (known) differences of the two methods in the conversion of minor oil/fat constituents, such as free fatty acids, wax esters and sterol esters. In case of FAME analyses, a hot injection is to be preferred over a cold injection. The RSDs of the peak areas were 1.5% for the major fatty acids to 11% for peaks that were just above the noise level. The detection limit were approximately 0.03%.

Keywords: Fats; Oils; Fatty acid methyl esters; *Cis*/*trans* methyl esters

1. Introduction

In the food industry, the preparation of oil and fat samples to enable the determination of the fatty acid composition (as their methyl esters or FAMEs), is one of the most frequently performed procedures. Next to the overall FAME composition, the *cis*/*trans* distribution is also important because of health aspects associated with *trans* fatty acids such as high cholesterol levels and heart diseases [1]. The currently most frequently performed so-called BF_3 method [2–6] for FAME and CTME analysis requires a relatively simple but laborious and time-consuming manual sample preparation. There is, therefore, a distinct need for a user-friendly, automated FAME/CTME preparation method based on the use of a robotic sample preparation system.

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The popular BF_3 procedure is, unfortunately, not a obvious choice if automated FAME sample preparation is the goal, since it involves heating to boiling point, the use of condensers and, also, large amounts of reagents. Alternative approaches to achieve methylation in a methanol medium, with the aid of acidic and/or alkaline catalysts, include the TMSH (trimethylsulfonium hydroxide) procedure [3], treatment with sulphuric acid or potassium hydroxide in methanol [2], and treatment with sodium methylate, $NaOCH_3$ [2,3]. Generally speaking, the various methods yield rather similar results. Minor differences between the methods, or within one method depending on the selected experimental conditions, mainly relate to differences in the methylation/transesterification of minor constituents of the fat or oil such as, e.g., the free fatty acids, wax esters and sterol esters. Unfortunately, most of these alternative methods are also time-consuming and some require boiling under reflux conditions. Upon closer scrutiny the $NaOCH_3$ method was considered the best option for use in an automated procedure.

In the overall procedure for FAME analysis, the chromatographic separation is also time consuming. Reducing the separation time therefore clearly would be attractive too. The only strategy to achieve this would be through the use of a column with a reduced inner diameter. Unfortunately, the highly polar stationary phases, such as CP-Sil 88, required for *cis*/*trans* separation cannot yet be coated reliably into columns with an inner diameter below approximately 200 μm; this due to Rayleigh instabilities [7,8].

In the present paper, a $NaOCH_3$-based robotic sample preparation procedure was designed and used for a variety of fat and oil samples. The results obtained were compared with those of the manual BF_3 procedure.

2. Experimental

2.1. Instrumentation

The entire process of sample preparation was performed on a Focus XYZ Sample Processing Robot (ATAS International, Veldhoven, The Netherlands). The GC injection interface was an Optic 2 programmable injector (ATAS International) containing a 80×3.4-mm I.D. liner with a glass frit located 15 mm from the bottom (ATAS International). The GC system was a HP6890 with FID detection (Hewlett-Packard, Wilmington, NC, USA). The capillary columns used were a 50-m×0.25-mm I.D. CP-SIL 88 for FAME with 0.4 μm stationary phase and a 100-m×0.25-mm I.D. CP-SIL 88 for FAME with 0.4 μm stationary phase, both delivered by Varian Chrompack (Middelburg, The Netherlands). Helium 5.0 (Hoekloos, Schiedam, The Netherlands) was used as carrier gas.

2.2. Focus XYZ sample preparation robot

The Focus XYZ sample preparation robot is a versatile system designed to process samples for routine GC and GC–MS analysis. The sample processor is based on a robotic XYZ arm with a motorised syringe, and comprises separate vial trays for samples, solvents and reagents, a syringe wash station and a heated sample agitator for mixing. Control is via a local module or a PC with software running under Windows. When using the PC software control the system can be user-programmed to emulate procedures commonly used in sample preparation for chromatographic analysis. Sample preparation procedures such as liquid–liquid extraction and derivatisation can be selected, programmed and performed in an automated manner to meet specific analytical requirements. The entire operation can be easily observed and any changes to the programme can be readily made. The Focus is located on top of the GC system and requires no additional bench space.

2.3. Chemicals

Solutions of fats and oils were prepared in *n*-heptane (Merck, Darmstadt, Germany). A saturated solution of sodium methylate (Merck) was prepared in LiChrosolv-grade methanol (Merck) and stored at 4°C.

2.4. Procedure and parameters

The BF_3 procedure was performed according to

the AOCS Official method [4]. From the final extract 1 ml was transferred to an autosampler vial. From this vial 1 μl was injected in the hot split mode. The automated $NaOCH_3$ procedure was based to the method of Schulte and Weber [3]. In the Schulte and Weber method 10 mg fat are dissolved in 1 ml of petroleum ether. Next, 20–50 μl of a 2-mol/l sodium methylate solution in methanol are added and the mixture is shaken for 30 s. After 30 min about 100 mg of calcium chloride salt are added, and after brief shaking the mixture is centrifuged. The upper layer is then ready for injection. In the automated procedure designed here sample preparation and analysis are synchronised. This means that the sample preparation is performed 'just-in-time'. About 10 min before the GC is ready for the injection of the next sample, sample preparation of that sample in the sample queue is started. This sample is then ready for injection at the moment the GC returns to the ready status. The total sample preparation, inclusive of weighing and heptane addition (2 min) takes 12 min.

Cis/*trans* FAME, i.e., CTME, separation requires careful selection of the isothermal oven temperature. Therefore, the optimum column temperature was determined using the procedure described by the AOCS [9]. For the 100-m column the isothermal temperature found for optimum separation was 176°C. For the CTME analyses the temperature was held at this value for 35.0 min. After elution of the last peak of interest the oven was rapidly (50°C/min) heated to 220°C and held at that temperature for 5.0 min to bake out the capillary column. During the run the carrier pressure was 467 kPa. For the 50-m column the whole analysis was performed isothermally at 176°C with a column head pressure of 150 kPa.

3. Results and discussion

In the process of setting up the automated $NaOCH_3$ transesterification it was found that a few minor changes to the procedure described by Schulte and Weber were necessary. Firstly, it is difficult to accurately weigh 10 mg of the oil/fat sample into a 2-ml autosampler vial. To make sure that the amount of $NaOCH_3$ added is sufficient, also if the sample amount exceeds 10 mg, 100 μl of the $NaOCH_3$ solution were added to the sample instead of the

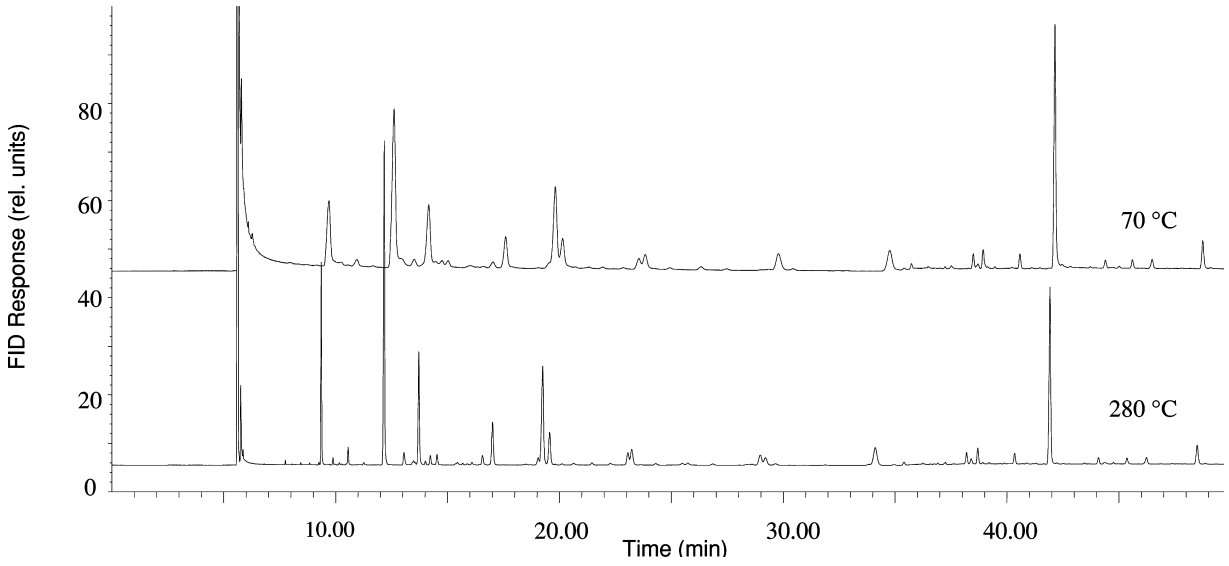

Fig. 1. GC–FID chromatograms of fish oil CTME analysis after sample preparation by the automated $NaOCH_3$ method. Upper trace: cold split injection at 70°C. Lower trace: hot split injection at 280°C. Column: 100 m×0.25 mm I.D. CP-SIL 88 for FAME with 0.4 μm stationary phase.

20–50 μl as described in the quoted procedure. Secondly, the Schulte and Weber procedure uses a reaction time of 30 s under shaking. The shaking time was increased to 60 s just to make sure that the reaction would always proceed to completion. In the original procedure, finally, $CaCl_2$ was added to the sample to improve phase separation and to remove traces of water that could affect the stability of the sample. In our experiments phase separation was found to be fast. The needle penetration depth was adjusted to ensure that the clear top layer of the reaction mixture was sampled. Moreover, as the sample was analysed immediately after preparation there was no need for stabilisation: any delay that could cause slow saponification of the FAMEs is avoided.

3.1. Hot versus cold injection

FAME samples can cover a wide range of boiling points (C_4–C_{24}). To avoid discrimination problems in the analysis of such samples it is often better to perform a cold injection rather than of a hot sample introduction. To study the difference between a hot and a cold injection all samples prepared using the sodium methylate method were injected both at 70°C (temperature-programmed cold split) and 280°C (isothermal or hot split). The overall results of the analyses showed that, in case of CTME analyses, a hot injection is to be preferred over a cold injection. The CTME separation has to be performed at an isothermal oven temperature. This means that — contrary to the situation in temperature-programmed GC — the slightly larger band width caused by the programmed heating of the injector cannot be improved by refocusing on the GC column. Fig. 1 shows chromatograms of a hot and a cold split injection of a $NaOCH_3$-prepared oil. The resolution obtained using hot split injection clearly is superior. By using a programmed GC oven, in contrast with the AOCS, and/or the use of a more polar solvent

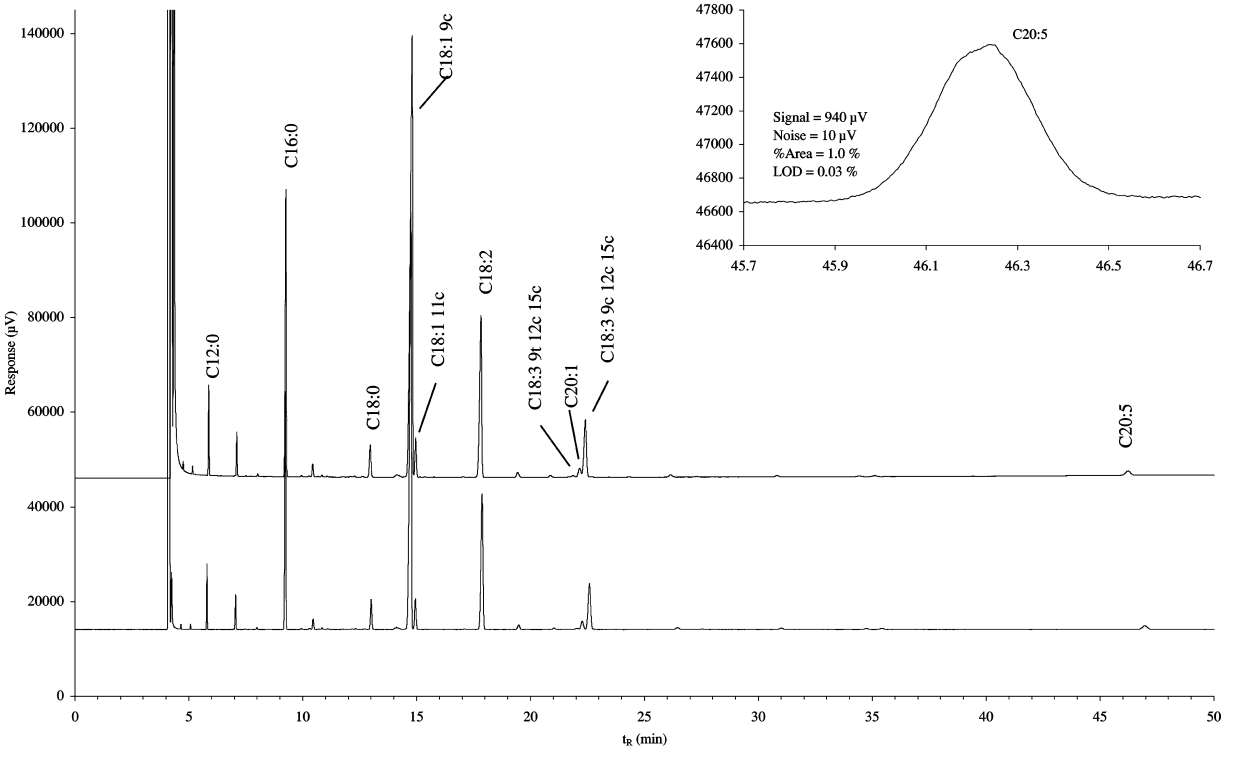

Fig. 2. GC–FID chromatograms of fish oil CTME analysis. Lower trace: FAMEs prepared by the manual BF_3 method. Upper trace: FAMEs prepared by the automated $NaOCH_3$ method. The insert is of the $C_{20:5}$ peak of the $NaOCH_3$ prepared sample. Column: 50 m×0.25 mm I.D. CP-SIL 88 for FAME with 0.4 μm stationary phase.

will overcome the band broadening [10]. However, this was not tested within this project.

3.2. BF_3 versus $NaOCH_3$ method

To be able to check the performance of the automated $NaOCH_3$ method, 29 samples covering a range of raw and processed vegetable oils and oil/fat blends widely differing in FAME composition, *trans* content, etc., were prepared and analysed using the standard BF_3 method. In addition to the vegetable oils, a fish oil sample was also included in the study. Subsequently, the samples were reanalysed using the automated $NaOCH_3$ method and the two sets of data were compared. Fig. 2 shows chromatograms of a sample prepared by the BF_3 and the automated $NaOCH_3$ method. The quantitative results of the two methods are compared in Fig. 3. From the linear relationship which is observed with $Y(BF_3)=1.0066\ X(NaOCH_3)+0.0042$ and $R^2=0.998$ it is evident that the experimental results are closely similar for all samples. The slope of the line is very close to unity indicating that the methods yield identical results. The somewhat larger discrepancy observed for the point marked by an asterisk is probably due to the fact that the BF_3 method also converts the free fatty acids (FFAs) into methyl esters, whereas these compounds are not methylated by the $NaOCH_3$ method [2]. Because most edible fat/oil samples contain only small amounts of FFAs, this is not a serious problem. This is even more true because the composition of the FFAs generally reflects that of the fatty acid composition of the triglycerides, consequently, no further attention was devoted to this aspect.

In Fig. 4 the results of the two methods are compared at the level of the individual FAMEs. The figure shows that for the major peaks in the chromatograms, e.g., C16:0 and C18:1 c, the agreement is better than for the minor peaks such as, e.g., C12:0, C16:1 c and C18:1 t. Most likely this reflects problems encountered with peak integration for peaks close to the noise level and not of the analytical procedures themselves. Again, the general agreement is satisfactory with all relative areas being in the range of 0.75–1.25. Except for the C12:0 all variations were random. The systematic deviation

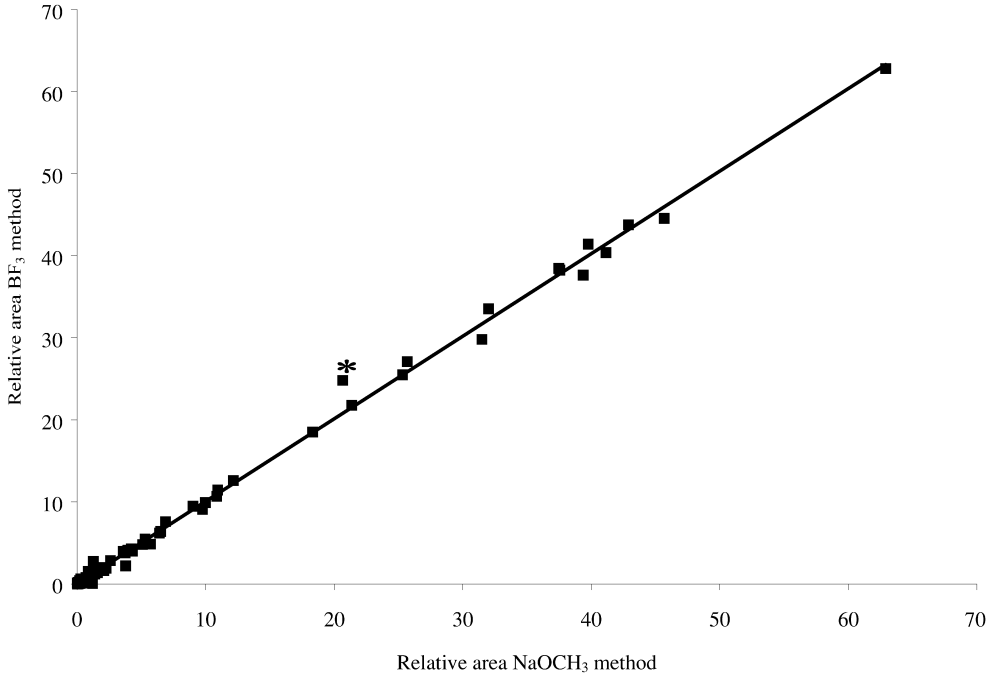

Fig. 3. Comparison of the relative peak areas obtained by GC–FID for BF_3 versus $NaOCH_3$ FAME sample preparation. The graph contains the data of 29 oil/fat samples containing fatty acids with from six to 24 carbon atoms and widely differing in, e.g., *trans* content.

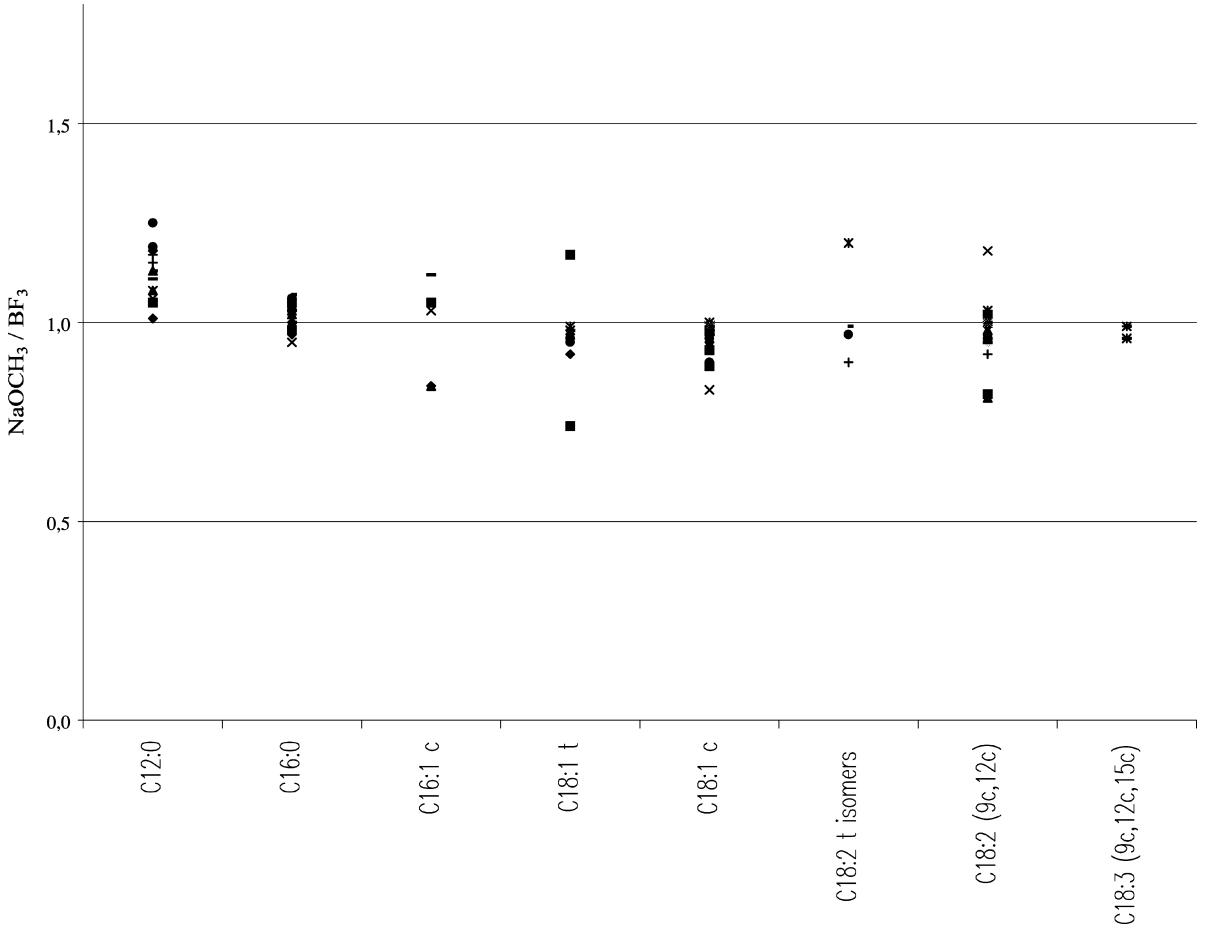

Fig. 4. Comparison of the areas of selected FAME peaks recorded after BF_3 and $NaOCH_3$ FAME sample preparation.

found for C12:0 might be a result of volatility losses in the BF_3 method.

3.3. Method performance

Three other characteristics of the newly implemented analytical procedure were assessed, repeatability, limit of detection and robustness. In the repeatability studies a fish oil sample was analysed (sample preparation and chromatography) nine times using the automated $NaOCH_3$ method. The RSDs of the relative peak areas were in the range of 1.5% for the major fatty acids to 11% for the peaks that were just above the noise level. For a complex sample as a fish oil this is clearly acceptable. The detection limits were determined from minor peaks present in the chromatograms. As an example, the insert in Fig. 2 shows the peak of C20:5 which is present at 1%. At a signal-to-noise ratio of 3:1, the detection limit was found to be 0.03%, which meets the desired range of 0.02–0.05%. During the study more than 100 samples were analysed without the need to change the injector liner and without encountering any instrumental problems. In other words, the automated method is robust and reliable.

4. Conclusions

The proposed automated and robot-based auto-

mated $NaOCH_3$ procedure for FAME/CTME sample preparation of fat and oil samples is about 4-fold faster (less than 15 min versus ca. 1 h) than the standard BF_3 method. This makes it relevant to similarly reduce the GC run time, i.e., to devote due attention to developing 50-μm I.D. columns coated with highly polar stationary phases.

The experimental results are closely similar, with small differences being due to the presence of, usually minor amounts, such as FFAs, which are methylated by the BF_3 method, but not converted by the $NaOCH_3$ transesterification. The automated $NaOCH_3$ method effects considerable cost-savings because of the reduced manual operator time and the increased sample throughput. The automated system, which is robust and user-friendly, is presently being used for the analysis of large numbers of real-life samples.

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