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Talanta 64 (2004) 1329–1334
Talanta
www.elsevier.com/locate/talanta

Developing an on-line derivatization of FAs by microwave irradiation coupled to HPLC separation with UV detection

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Received 23 March 2004; received in revised form 6 May 2004; accepted 11 May 2004
Available online 28 July 2004

Abstract

The development of analytical methods for routine simultaneous identification and quantification of carboxylic fatty acids (CFAs) are required in different fields, such as, pharmaceutical cosmetics, food products and formulations of water–microemulsion–oil systems. Determination of CFAs has been developed mainly by gas chromatography (GC). As an alternative to GC, liquid chromatography (LC) has better sensitivity and selectivity. However, most CFAs show no useful absorption in ultraviolet–violet (UV–Vis) region, one of the more used detection technique in high-performance liquid chromatography (HPLC). In order to allow the use of UV–Vis detection, the use of pre-column derivatization has been reported to increase sensitivity and selectivity. Therefore, establishment of a simpler and faster on-line method with complete separation is needed for the screening of large numbers of samples. 2,4-Dinitrophenylhydrazine (2,4-DNPH.), benzoil chloride (BC), and phenylhydrazine (PH) were used for derivatization of different FAs by microwaves radiation (MW). After the on-line derivatization, products were separated and quantified by HPLC. Reactor coil was placed inside of microwaves oven at 450 W. Parameters as flow, amount of reagents, irradiation time, and chromatographic conditions were optimized. The continuous analysis using the MW–HPLC–UV system provided high sensitivity and reduced both the amount of reagent used and the analysis times. This proposed method can be used for the routine analysis of FAs contained in water–microemulsion–oil systems, to quantify the total acid fraction in each phase.

Keywords: Carboxylic acids; Derivatization; On-line system; Microwave irradiation

1. Introduction

Fatty acids (FAs) are widely used in industries [1–3], and they are common compounds found in foods, and pharmaceuticals products. FAs can be ionized by a change of pH; this acid property together with a long chain makes them an amphiphilic compound, which is very useful for natural microemulsion generation. In these microemulsions, the oil and water phases are immiscible, but the presence of a third component with amphiphilic properties, such as the FA, reduces the immiscibility.

In the seventies, the enhanced oil recovery application of surfactant–oil–water system (SOW) drove an outstanding research effort that resulted in a considerable improve of the knowledge concerning the phase behavior of the systems [4–6]. So, methods to determine and to quantify all species presents in the SOW systems are desirable.

The determination of FAs has been reported by gas chromatographic (GC) and liquid chromatographic (LC) [7–14]. High performance liquid chromatographic (HPLC) offers high sensitivity and selectivity, but most FAs have poor absorption in the ultraviolet–violet (UV–Vis) regions the wider detection system used in LC. Therefore, wide variety of esterification methods for carboxylic acids as well as condensation of carboxylic acids with alcohols under acidic conditions or reaction of carboxylic acids with alkyl halides under basic conditions have been reported in order to increase absorption in the UV–Vis region [15–22]. Phenyl-

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doi:10.1016/j.talanta.2004.05.022

hydrazine (PH), 2,4-dinitrophenylhydrazine (2,4-DNPH), benzoil chloride (BC) reagents have been reported for FAs derivatization [23,24].

Derivatization techniques for HPLC analysis have received special attention because they enable highly sensitive detection of these compounds by bonding a chromophore that results in products with strong UV absorption. There are two alternatives for FAs derivatization, by reaction before or after chromatographic separation, using an off-line or on-line system. The pre-column derivatization [19,23,24] is used more frequently than the post-column [19,23,24] derivatization in order to increase sensitive detection. FAs derivatization required some minimum reaction condition to produce the product. Most of the derivatization reaction reported the used of solvent and heating in a boiling water to get the product [25,26]. On the other hand, microwave (MW) heating can advantageously replace classical heating because it allows the irradiation of the reaction mixture in continuous or no-continuous system; at the same time, it permits the efficient control of the given energetic power. For all these reasons, microwave irradiation can be employed to accelerate chemical reaction and rate enhancements of up to 1000-fold over conventional conditions. Experimental conditions, such as the amount of reagent, reaction temperature, and reaction time, are sometimes critical for high reaction efficiency and to avoid side product formation; the cleanup and derivatization procedures are sometimes complicated and time-consuming. In recent years, a number of methods for the preparation of carboxylic esters from carboxylic acids were reported utilizing microwave irradiation conditions [27,28]; derivatized step was previous to the separation one. The main objective of this work was the development of an on-line system for the derivatization of FAs using microwave irradiation and coupling it to HPLC–PDA system for FAs separation. This proposed method can be used as routine procedure in the analysis of FAs in SOW systems.

2. Experimental

2.1. Apparatus

High-performance liquid chromatography separations were performed with a set-up from Waters Corporation consisting of a 510 pump and a U6K injector valve with a sample loop of 10 μL, photodiode array detector (PAD), model 996 coupled to a PC loaded with Millennium software. The RP-18 bonded silica column (250 mm × 4.6 mm i.d.) was purchased from Merck. On-line derivatization system consisted of an isocratic high pressure pump model 510 from Waters, a Rheodyne injection valve model 5020 and switching valve model 5011 from Supelco. A domestic microwave oven (Kenmore) equipped with a magnetron of 2450 MHz with a nominal maximum power of 400 W as marketed was used. The coil reactor was introduced through the vent holes of the microwave oven in order to avoid drilling of the walls. Fig. 1 shows manifold used.

2.2. Solvents and reagents

Methanol (MeOH) and acetonitrile (ACN) used were of HPLC grade from Baker Chemicals. All solvents were

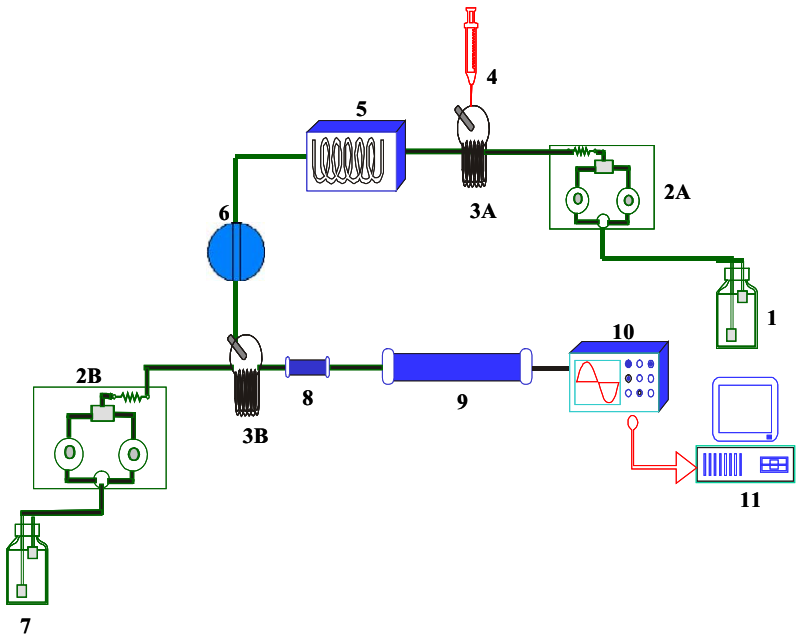

Fig. 1. Schematic diagrams of on-line fatty acid derivatization–HPLC system. (1) ACN; (2A) and (2B) pump; (3A) 100 mL and (3B) 10 mL injection valve; (4) fatty acid plus reagent; (5) coil reactor, MW (450 W); (6) switching valve; (7) 92/8 MeOH/H_2O; (8) pre-column; (9) column RP-18; (10) PDA detector; and (11) PC process.

Table 1
Operational conditions for on-line derivatization

Reagent	Reagent concentration (mM)	Wavelength (nm)	Acid (mM)	Coil Rx (μL)	Flow pump A (mL min^{-1})	Flow pump B (mL min^{-1})
2,4-Dinitrophenilhidrazine	1.00	310	0.0591	1000	0.8	1
Phenylhydrazine	1.99	254	0.0591	1000	0.8	1
Benzoil chloride	1.724	254	0.0591	1000	0.8	1

V_{inj} pump A = 10 μL; and V_{inj} pump B = 100 μL.

ultrasonically degassed. The studied samples were as follows: caprylic acid (C8), capric acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18), all provided by Merck. They are referred to as C*N*, where *N* indicates the number of carbon atom in the acid molecule. The reagents used to produce the derivates were phenylhydrazine, 2,4-dinitrophenylhydrazine, and benzoyl chloride (purity over 99%). They were supplied by Merck Company. Derivatization conditions are shown in Table 1.

2.3. Procedure

The continuous system developed for the on-line derivatization of FAs is depicted in Fig. 1. Initially FAs solution containing 0.0591 mmol of each acid in acetonitrile medium and derivatized reagent (BC, PH and 2,4-DNPH, respectively) filled the loop of injection valve (3A). By switching the injection valve, loop content was inserted into the acetonitrile carrier stream and driven to the reactor coil located inside the microwave oven. In this system, coil was wrapped around an assay tube. About 30 s after sample injection, when the sample plug was inside the reactor coil, the microwave oven was switched on at 400 W for 1 min. Due to the low power of the microwave used, not excessive heating was rinsed, and in this way, bubble formation was avoided. After derivatization process, the loop of injection valve (3B) was automatically filled and by switching it, the reaction products were separated by HPLC.

3. Results and discussion

3.1. Reaction conditions

The proposed method is based on the reaction of the carbonyl group from FAs with three different derivatized reagent, as it is shown in Fig. 2. Acetonitrile was used as reaction solvent. FAs derivatized present a strong absorption at wavelength showed in Table 1; those results are in agreement with the reports by Miwa [29]. Some authors [30] have reported that the best yield is obtained when the derivatization reagent is added in excess.

When reaction was carried out by conventional conditions as heating in a water bath (using 2,4-DNPH as a derivatized reagent), time consumption was 60 min at 60 °C. On the other hand, when the reaction was carried out with microwave irradiation, at 450 W, reaction was accelerated and rate enhancements of up to 10-fold occurred over the previous conditions; these results agree with the microwave benefits reported by other authors that were discussed before [25,26]. For this reason, microwave irradiation was selected for the derivatized step.

3.2. Developing the on-line system

In the on-line system, the exposure time is determined by the reactor volume and flow rate. Initially, a reactor coil of 130 cm length and 0.8 mm internal diameter was used, but broad peaks were obtained. When the internal diameter

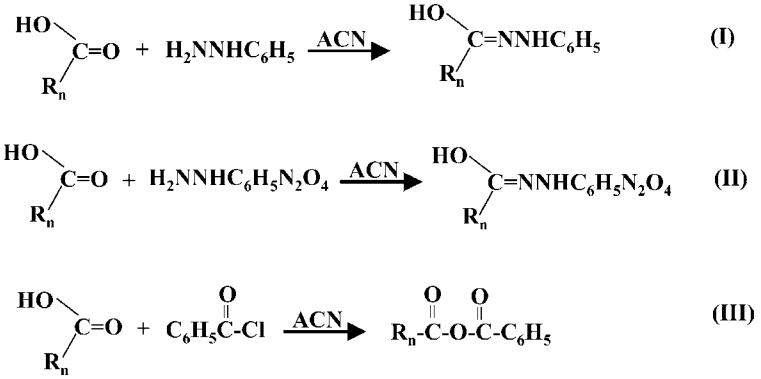

Fig. 2. General chemical reaction of long-chain fatty acid with the derivatized reagent. (I) Phenylhydrazone of fatty acid; (II) 2,4-dinitrophenylhydrazone of fatty acid; and (III) benzoil fatty acid ester.

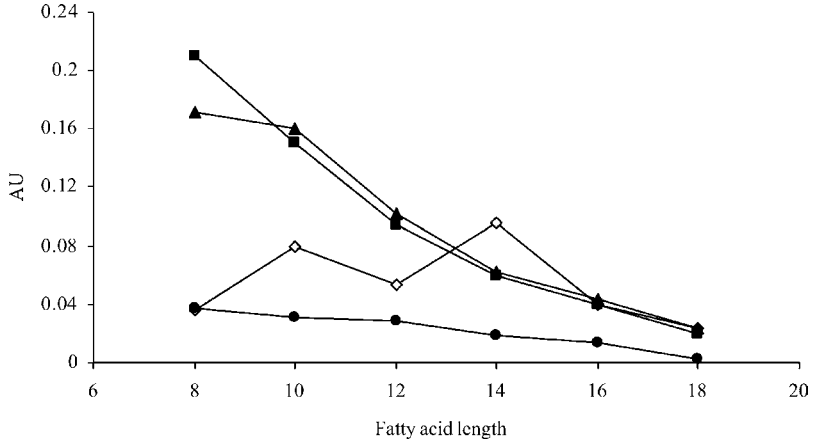

Fig. 3. Variation of intensity with the size-chain-length of FAs with: (◇), benzoil chloride; (▲), 2,4-dinitrophenilhidrazine; (■), phenilhidrazine; and (●), unreacted.

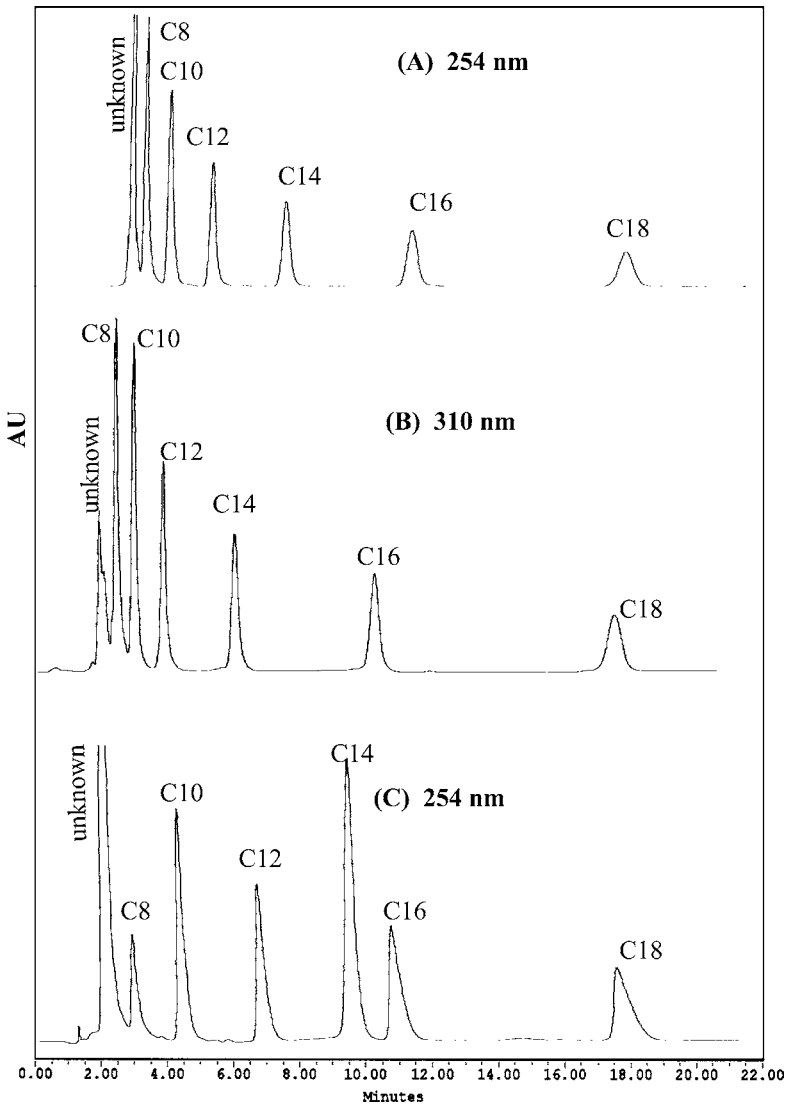

Fig. 4. Chromatograms of FAs derivatization using (A) phenylhydrazine, (B) 2,4-dinitrophenilhidrazine, and (C) benzoil chloride. Column: m-Bondapack C18 (250 × 4.6 mm i.d.). Mobile phase: 92/8 MeOH/H_2O.

was decreased to 0.1 mm, the peak obtained was thinner and higher than that obtained with the winner tubing; this better peak shape was due to the dispersion, which was reduced using 0.1 mm. The effect of the flow rate on the peak height was studied in the range of 0.1/1.5 ml min^{-1}. The peak height decreased with the increase of the flow rate. Taking into consideration the stability of the pump, peak shape and sampling time, the flow rate of the reagent carrier solution was adjusted to 1.0 ml min^{-1}.

The reactor coil was placed into a domestic microwave oven linked to the separation system (see Fig. 1); irradiation times from 1 to 60 s were used, and it was observed that after 40 s the signal intensity was almost constant. High flow rates were assayed to decreased analysis time, but with flow rates higher than 0.8 mL min^{-1}, smaller peaks were observed.

3.3. HPLC analysis conditions

The separation of six FAs was attempted on a C18 column (5 mm), which is widely used for this kind of separation [31,32]. At the beginning, pure MeOH was used, but poor resolution was obtained. When H_2O was added to the mobile phase, resolution improved; the optimum proportion was 92/8 (MeOH/H_2O). This mobile phase was appropriate for the analysis of the FAs with the three reagents derivatized because time is saved in the chromatographic analysis.

3.4. Derivatized reagent

It is well known that most of the solvents used as mobile phase in HPLC present a strong absorption, which can interfere with acid peaks at 214 nm; however, when acids are derivatized, sensitivity and selectivity increase. Signal intensity of FAs without derivatization after their separation was compared with their signal intensity after using BC, PH and 2,4-DNPH derivatization reaction. As it is shown in Fig. 3, it is clear that when the derivatization step was added to FAs, analysis by HPLC–PDA sensitivity increase until seven times when PH was used. In general, peak tendency showed an inverse relationship between product absorptions and their number of carbon atoms; it means that absorption decreased when the size-chain-length increased as can be seen in Fig. 3.

Of the three different reagents, PH showed the higher intensity for the octanoic acid; however, for the longer acids, PH and 2,4-DNPH present similar behavior as can be seen in Fig. 2. Therefore, the use of 2,4-DNPH is better because the derivatized product has an strong absorption at 310 nm, which presents less interferences than the 254 nm used with the PH product. The main advantages of 2,4-DNPH reaction was that the amount of unreacted reagent was very low as can be seen in Fig. 4. In this figure, the baseline presents some small peaks that can be attributed to by-products. It was found that the use of BC compared with the other two reagents do not present any advantages because BC products showed no linearity as can be seen in Fig. 4. Moreover, absorption of derivatized products is low; it was just three times higher than that without derivatization. The precisions (relative standard derivation, RSD) and accuracy (relative error, RE) of the method were studied based on the peak-area ratios for the analysis of each FAs at 0.059, 0.079 and 0.119 mM. In all cases, RSD and RE below 4 were obtained.

4. Conclusions

Adding derivatization step to FAs analysis by HPLC–PDA increased sensibility almost seven times. Additionally, acid derivatization allows to used 254 and 310 nm as wavelength for detection. From the comparison of the reagents used, it could be said that the better one was 2,4-DNPH. Microwave irradiation, at 450 W, was more efficient for the derivatization than the conventional conditions such as heating in a water bath. Reaction time can decrease almost 10 times. The developed continuous analysis using the MW–HPLC–UV system provided high sensitivity, reduced the amount of reagent used and permitted to obtain shorter analysis times.

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