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Differently Saturated Fatty Acids Can Be Differentiated by ^{31}P NMR Subsequent to Derivatization with 2-Chloro-4,4,5,5-tetramethyldioxaphospholane: A Cautionary Note

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ABSTRACT: The analysis of free fatty acid (FFA) mixtures is a very important but, even nowadays, challenging task. This particularly applies as the so far most commonly used technique—gas chromatography/mass spectrometry (GC/MS)—is tedious and time-consuming. It has been convincingly shown (Spyros, A.; Dais, P. *J. Agric. Food Chem.* **2000**, *48*, 802−5) that FFA may be analyzed by ^{31}P NMR subsequent to derivatization with 2-chloro-4,4,5,5-tetramethyldioxaphospholane (CTDP). However, it was also indicated that differently unsaturated FFAs result in the same ^{31}P NMR chemical shift and cannot be differentiated. Therefore, only the overall fatty acid content of a sample can be determined by the CTDP assay. In contrast, we will show here by using high-field NMR (600 MHz spectrometer, i.e., 242.884 MHz for ^{31}P) that the CTDP assay may be used to differentiate FFAs that have pronounced differences in their double bond contents: saturated fatty acids (16:0), moderately unsaturated (18:1, 18:2), highly unsaturated (20:4), and extremely unsaturated fatty acids (22:6) result in slightly different chemical shifts. The same applies for oxidized fatty acids. Finally, it will also be shown that the CTDP derivatization products decompose in a time-dependent manner. Therefore, all investigations must adhere to a strict time regime.

KEYWORDS: *free fatty acid, ^{31}P NMR, CTDP derivatization*

1. INTRODUCTION

Free fatty acids (FFAs) play an important role in the organisms of vertebrates, although their concentration must not exceed a certain limit due to their detergent properties.[1] FFAs are, for instance, particularly important in hepatocytes (liver cells), where they can be either converted into triacylglycerols (storage form of an excess of energy) or oxidized to provide energy for the organism. Additionally, FFAs represent the educts of the synthesis of phospholipids and messenger molecules such as prostaglandins and leukotriens.[2] Hepatic steatosis (i.e., the abnormal retention of lipids within liver cells leading to the pathologic fatty liver) is becoming a significant public health concern in Western societies and FFAs are assumed to be crucially involved in the related pathogenesis.[3]

In addition to this significant physiological interest, the FFA content is also of considerable interest in nutrition and food research,[4] for instance in vegetable oils and fruit juices.[5] Therefore, the detailed—quantitative and qualitative—analysis of the FFA patterns is very important.

Gas chromatography/mass spectrometry (GC/MS) is even nowadays the most commonly used technique of FFA analysis[6,7] because (using traditional electron ionization (EI)) quantitative information can be derived directly from the spectra. However, this method is tedious and time-consuming, because separation and derivatization steps (methylation or silylation) are required to enhance the volatilities of the individual FFAs.[8] In order to overcome these drawbacks, there have been many efforts to use soft ionization MS such as electrospray ionization (ESI)[9] or matrix-assisted laser desorption and ionization (MALDI).[10,11] MALDI MS is particularly simple and, thus, capable of high throughput screening in "omics" initiatives. Unfortunately, however, the considerable "matrix" background[12] is a serious problem and the limited stability of FFAs under high vacuum conditions also affects the accuracies of the measurements. Therefore, none of these methods has so far been practically used to investigate FFA patterns.

Although nuclear magnetic resonance (NMR) spectroscopy suffers from reduced sensitivity in comparison to MS techniques as well as enzymatic assays,[13] NMR is a powerful method to study the compositions of complex mixtures. Although the proton (^{1}H) is the most sensitive nucleus, the relatively small chemical shift range (about 10 ppm) causes problems regarding signal overlap, that is, the achievable resolution is limited. This particularly applies for mixtures that consist of very similar compounds—such as in the case of FFAs due to the large alkyl chains. This limits the application of ^{1}H NMR, although some work has been performed in the field of FFAs.[14] In contrast, ^{13}C NMR is characterized by a much wider range of chemical shifts and is basically capable to differentiate even complex mixtures of FFAs.[15] Unfortunately, however, the sensitivity of ^{13}C is much smaller in comparison to ^{1}H and thus, its application is limited to concentrated samples. Additionally, there are many concerns regarding quantitative aspects of ^{13}C spectra: the significant T_1 relaxation times of the carbon nucleus require considerable measuring times because a long delay between two pulses is necessary to avoid saturation effects. Finally, the "Nuclear Overhauser Effect" (NOE) which is observed if decoupling (to simplify the spectra and to achieve an improved signal-to-noise ratio) is used, may affect the

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intensities of the individual carbon resonances to a different extent.[16]

The ^{31}P nucleus is a classical "good" NMR nucleus, because it exhibits high sensitivity and is present in a natural abundance of 100%. Additionally, the integral intensities of ^{31}P NMR spectra are affected to a lesser extent by NOE effects because the gyromagnetic ratios (γ) of ^{1}H and ^{31}P are quite similar - at least if compared to ^{13}C.[16] Of course, native FFAs as such are not detectable by ^{31}P NMR, because they do not possess a ^{31}P nucleus. However, FFAs can be easily derivatized into compounds detectable by ^{31}P NMR. Spyros and Dais have shown in an elegant work[17] that 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane (CTDP) is a suitable reagent that enables the complete conversion of −O-H-acidic compounds (such as FFAs, but also many other compounds, for instance aldehydes, mono- and diacylglycerols, etc.) into substances that can be characterized by means of ^{31}P NMR. Since this study[17] was aimed at the investigation of virgin olive oils, particular attention was paid to the analysis of di- and monoacylglycerols, free fatty acids and aldehydes that are all important alteration products of vegetable oils. A particular advantage of this approach (in comparison to GC/MS) is that it can be very easily performed: with the exception of the CTDP addition no sample workup (time-consuming derivatization or separation) is necessary and this minimizes the time that is required to analyze a given sample. These advantages have also been recently reviewed.[18]

It was also stated in previous work[17] that the fatty acyl compositions of the investigated oils and, thus, the structures of the released FFAs have only negligible effects on the observed chemical shifts. This result is not very surprising, as mixtures of very similar FFAs (oleic acid (18:1), linoleic aicd (18:2) and linolenic acid (18:3)) were investigated. However, the interest in these FFAs resulted from the author's interest in vegetable oils, where these fatty acyl residues are most abundant.[19] In contrast, many cellular or tissue samples contain huge amounts of higher unsaturated FFAs such as arachidonic (20:4) and docosahexaenoic acid (22:6). Surprisingly, the related ^{31}P NMR chemical shifts resulting from the derivatization of these FFAs with CTDP have not yet been evaluated. We will show here that the observed slight differences in the ^{31}P NMR chemical shifts are sufficient to allow a coarse differentiation of the individual FFAs: as already described in the context of phospholipids, at least saturated, moderately unsaturated and highly unsaturated FFA result in different ^{31}P NMR chemical shifts.[20]

Finally, we will also show that the obtained ^{31}P NMR spectra show time-dependent changes that are presumably caused by oxidation processes of the unsaturated FFAs and the lability of the three-valent phosphorus. Thus, it is concluded that a carefully controlled time-regime is mandatory to achieve reliable data. This is particularly important if highly diluted samples (requiring a significant number of scans) have to be investigated.

2. MATERIALS AND METHODS

2.1. Chemicals. All free fatty acids (myristic (14:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4) and docosahexaenoic (22:6) acid) and the (MALDI) matrix 1,8-bis-(dimethylamino)-naphthalene (DMAN)[11] were obtained from Sigma-Aldrich (Taufkirchen, Germany) in the highest commercially available quality and used as supplied. The same applies for the chemicals for NMR spectroscopy (2-chloro-4,4,5,5-tetramethyl-dioxaphospholane (CTDP), chromium(III) acetylacetonate ($Cr(acac)_3$), cyclohexanol, deuterated chloroform ($CDCl_3$) with an isotopic purity of 99.6%) as well as all solvents (chloroform, methanol and pyridine).

2.2. Sample Preparation. All FFAs were used as supplied, that is, without further purification and in a concentration of 100 mg/mL in $CDCl_3$ dried over molecular sieve; 62.5 μL (10 mg/mL final concentration) of this solution were mixed (directly in a 5 mm NMR tube) with 500 μL deuterated chloroform, 62.5 μL of a 1:5 diluted stock solution (in $CDCl_3$) of 0.6 mg $Cr(acac)_3$ and 1.35 mg cyclohexanol prepared in 10 mL of dry solvent (pyridine and $CDCl_3$ = 1.6:1 (v/v)). Thus, the final concentration of the fatty acid in the NMR tube (total volume 640 μL) was about 10 mg/mL (if not otherwise stated). This high concentration was used to be able to perform all NMR measurements as quickly as possible. At least 15 μL of 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane (CTDP) were added at ambient temperature. The concentration of CTDP is not important; however, it must be in excess of all the fatty acids of interest. NMR investigations were performed about 10 min after the addition of the CTDP.

2.3. MALDI-TOF Mass Spectrometry. The purities of all used FFA samples were checked by MS prior to NMR characterization: Stock solutions of the individual FFAs (0.1 mg/mL) were prepared in chloroform and diluted 1:1 (v/v) with the DMAN matrix (10 mg/mL in ethanol) as previously described.[11,10] Selected oxidized FFAs were also investigated: a dried film of the FFAs was oxidized by exposition to air for 12 h and the FFAs were afterward redissolved in $CHCl_3$. The FFA/matrix mixtures (about 0.50 μL each) were subsequently applied onto a gold-coated standard aluminum MALDI target. Thus, the absolute amounts on the target were in the range of about 200 fmol. All mass spectra were acquired on an Autoflex I MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in the reflector mode. The system utilizes a pulsed 50 Hz nitrogen laser emitting at 337 nm, and the extraction voltage was 20 kV. The laser fluence was set about 10% over threshold and the pulse delay (extraction time) was adjusted between 20 and 100 ns in order to give optimum results. All measurements were performed in the negative ion mode and the high vacuum in the ion source was 5×10^{-7} mbar. All mass spectra were processed by using the instrument software "Flex Analysis 3.0" (Bruker Daltonics, Bremen).

2.4. NMR Spectroscopy. All NMR measurements were performed on a Bruker DRX-600 spectrometer operating at 242.94 MHz for ^{31}P using a 5 mm direct broadband probe at 37 °C; this temperature gave in our hands the best resolution and is also applicable if phospholipids within the sample have to be investigated. To eliminate nuclear Overhauser (NOE) effects, the inverse gated decoupling sequence was used.[16] Typical spectral parameters were as follows: 90° pulse, sweep width 8 kHz, relaxation delay 5 s, 16k data points, 128 scans. No window functions were used to process the free induction decays (LB = 0).

Chemical shifts were referenced to the reaction product of the CTDP with internal cyclohexanol providing an intense ^{31}P resonance at 142.5 ppm.[17]

3. RESULTS AND DISCUSSION

The assay suggested by Spyros and Dais[17] is simple and elegant. The derivatization of all acidic compounds (which is illustrated in the upper part of Figure 1 for FFAs as an example) can be easily accomplished by using an excess of CTDP. There are, however, three important prerequisites: (1) The sample should be free of water and other impurities with acidic protons (e.g., alcohols that are commonly used as stabilizers of $CHCl_3$), as such compounds are also capable of reacting with CTDP. For instance, the resonance of the pure CTDP (about 176 ppm in $CDCl_3$)[21] is converted into another resonance at about 132.2 ppm in the presence of water.[17] It is also important to avoid the presence of ethanol in the $CDCl_3$. As chloroform decomposes under generation of phosgen ($COCl_2$), ethanol is commonly added in order to convert this toxic compound into less

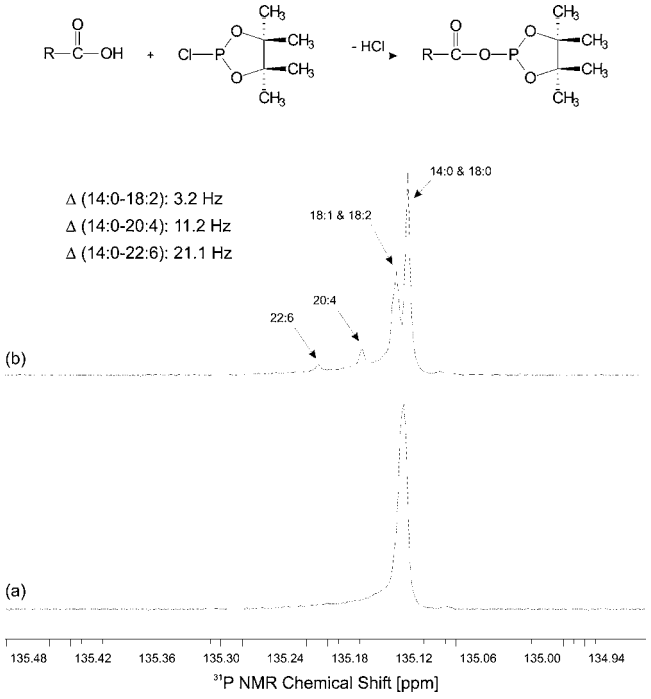

Figure 1. ^{31}P NMR spectra of mixtures of free fatty acids subsequent to derivatization with 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane. In (a) a mixture between 18:1, 18:2 and 18:3 (3/2/1, *w/w/w*) was investigated, while in (b) a mixture between 14:0, 18:0, 18:1, 18:2, 20:4 and 22:6 (6/4/4/3/2/1, *w/w/w/w/w/w*) is shown. The different amounts of the FFAs were chosen in order to enable direct assignments of the individual resonances. As the shift differences are very small, the differences between myristic acid and some selected FFAs are expressed in Hertz and shown in trace (b). Please note that fatty acids with a similar content of double bonds cannot be differentiated by ^{31}P NMR. The applied derivatization reaction between FFAs and CTDP is shown at the top of the figure.

harmful products. However, no ethanol must be present in the used solvents. In contrast, the presence of phospholipids such as phosphatidylcholine (PC) is not a problem since they do not react with CTDP. (2) The CTDP must be in sufficient excess of the compounds (here the fatty acids) that are to be derivatized. Otherwise, not all compounds of interest would be completely derivatized, and this would make quantitative investigations impossible. The presence of an excess of the CTDP is not a problem, because its resonance is outside the spectral range of interest.[17] This also applies for potential oxidation products of the CTDP, since penta-valent phosphorus exhibits its resonance close to zero ppm and, thus, far from the spectral range of three-valent phosphorus. This is helpful and no handling of the samples under careful exclusion of oxygen is required. (3) The time between two excitation pulses must be sufficiently long to allow complete relaxation, since otherwise saturation effects would also play a significant role.[16]

Although it was indicated that all FFAs result in exactly the same chemical shift,[17] this is not completely true which is illustrated by two different spectra shown in Figure 1. The spectrum shown in trace (**1a**) represents a mixture between the FFAs 18:1, 18:2 and 18:3 subsequent to derivatization with CTDP. These FFAs were selected because they are extremely abundant in vegetable oils[19] and have, thus, been already investigated in the past.[17] It is obvious that a mixture of these three FFAs results in the same ^{31}P NMR chemical shift and therefore the individual FFAs cannot be differentiated. In contrast, a more complex mixture was investigated in trace (**1b**). From this spectrum it is obvious that differences in the content of double bonds lead to changes in the chemical shifts. For instance, saturated fatty acids (14:0/18:0), moderately unsaturated (18:1/18:2), highly (20:4) and extremely unsaturated fatty acids (22:6) can be differentiated by slight changes of the chemical shifts. All FFAs were used in different amounts to avoid assignment problems. It is obvious that an increased double bond content (and thus an enhanced electron density) is accompanied by a downfield shift of the corresponding ^{31}P resonance. This tendency is comparable to that observed in the case of phospholipids[20] and is useful to investigate the FFA composition of complex mixtures - although no very detailed analysis is possible but only a differentiation between saturated, moderately unsaturated and highly unsaturated compounds can be made. It should finally be noted that the presence of bulk membrane phospholipids such as phosphatidylcholine (PC) does not affect the analysis of free fatty acids: on the one hand, PC does not possess acidic groups that would be capable of reacting with the CTDP. On the other hand, the ^{31}P NMR chemical shifts of phosphorus(V) and (III) compounds are very different and, thus, no superposition occurs.[16] Although there are some phospholipids (for instance, lyso-phosphatidylcholine) with reactive hydroxyl groups, their concentration is normally extremely low and does not hamper the successful identification of the derivatization products of FFAs.

Additionally, it was also found that one must adhere to a strict time regime because the stability of the derivatized compounds is only limited due to the presence of three-valent phosphorus. This is illustrated in Figure 2, where the time-dependence of the ^{31}P NMR spectra of isolated palmitic acid subsequent to derivatization with CTDP was investigated;

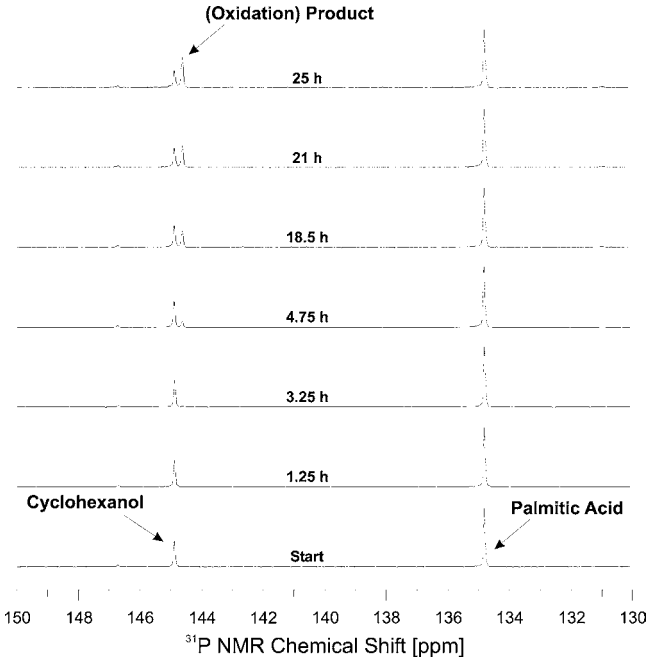

Figure 2. Time-dependent ^{31}P NMR spectra of a mixture between cyclohexanol (typical reference compound[17]) and palmitic acid subsequent to derivatization with 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane. The times after which the spectra were recorded are indicated in the individual traces. Please note that there are exclusively oxidation products of the cylohexanol standard but not the palmitic acid.

cyclohexanol was used as standard.[17] Although the peak intensities remain virtually identical over the first few hours, there are notable changes after this time. While the resonance of the derivatization product of palmitic acid does not exhibit any changes, major changes of the cyclohexanol as internal standard are obvious: an additional small resonance (labeled "oxidation product") appears close to the cyclohexanol standard at slightly lower ppm values. We have also tried to investigate the chemical structures of the "new" resonances by recording ^{1}H NMR spectra. However, this attempt was not successful: since neither the CTDP nor the pyridine in the solvent mixture was deuterated, the ^{1}H NMR spectra were dominated by these resonances and the detection of compounds present in comparably small amounts was impossible. Although the chemical reasons of the observed changes are not thus clear so far, one should keep carefully in mind that these changes aggravate the quantitative data analysis of highly diluted samples (requiring a significant number of scans and, thus, considerable measuring time), because the intensity of the standard does not remain constant.

The situation is even more complicated if the CTDP derivatization products of unsaturated fatty acids such as arachidonic acid (containing four double bonds and very abundant in porcine meat and generally in meat products[22]) are investigated (Figure 3). Here, an additional resonance (beside that of arachidonic acid at 134.8 ppm) is obvious at slightly smaller ppm values (at 130.9 ppm). It is very likely that this resonance corresponds to peroxidation products (particularly hydroperoxides) of arachidonic acid because these oxidation compounds possess an acidic -O−OH group that reacts with the CTDP: the same resonance is observed if artificially oxidized arachidonic acid is investigated by means of NMR (data not shown).

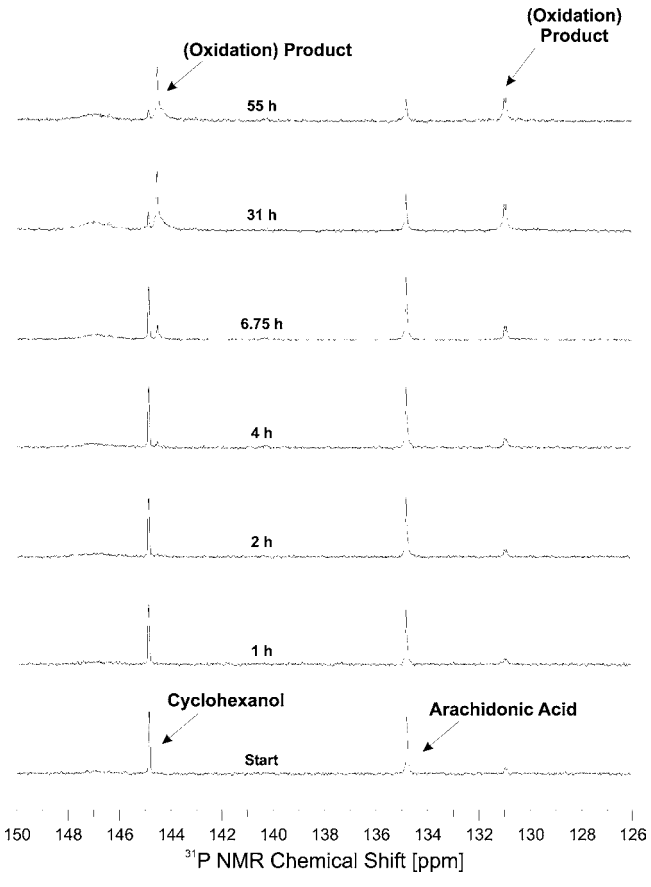

Figure 3. Time-dependent ^{31}P NMR spectra of a mixture between cyclohexanol (typical standard[17]) and arachidonic acid subsequent to derivatization with 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane. The times after which the spectra were recorded are indicated in the individual traces. Please note that there are oxidation products of the cylohexanol standard and the arachidonic acid.

Basically, there are two different oxidation products of FFAs: (hydro)peroxides, which are generated by the addition of oxygen, and aldehydes originating from a cleavage at the double positions.[23] It can be excluded that the resonance at 130.9 ppm is derived from aldehydes, since aldehydes subsequent to CTDP derivatization are detectable at a different chemical shift.[17] Therefore, hydroperoxides must be responsible for the arising of the additional resonance.

Finally, a more detailed investigation of the time-dependence of the spectra shown in Figure 2 was performed and the corresponding data are shown in Figure 4. It is obvious that

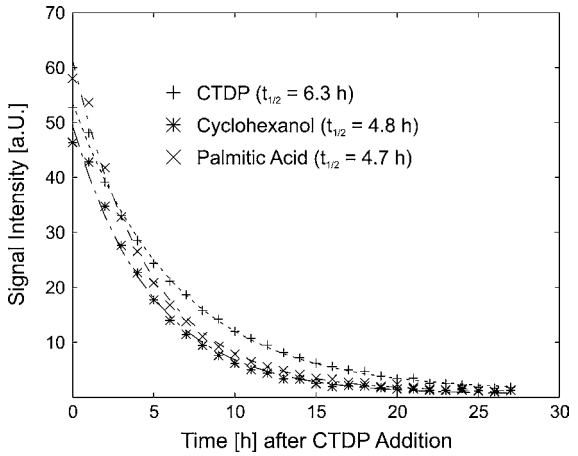

Figure 4. Quantitative evaluation of the data shown in Figure 2. The absolute intensities of 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane (176 ppm), and cyclohexanol (145 ppm) as well as palmitic acid (134.8) subsequent to derivatization with CTDP are plotted against time. "Zero" means the insertion of the sample into the magnet. ^{31}P NMR spectra were recorded after intervals of one hour. These data clearly indicate that quantitative information may be only obtained if the time-dependent changes are kept in mind. This is a particular problem if highly diluted samples are of interest.

there is a decay of both the derivatization product of the cyclohexanol (145 ppm) and the FFAs (134.8 ppm), and that the half-life of both processes is very similar -4.8 and 4.7 h, respectively. Since both compounds have completely different structures but possess the same element, the three-valent phosphorus, it is suggested that the intensity loss is caused by the oxidation into penta-valent phosphorus. The shifts of such compounds are outside the observed spectral window and a more detailed investigation of these processes was beyond the scope of this study. However, this assumption is supported by the fact that the intensity of the resonance of the used CTDP is also diminished and this process is characterized by a half-life of 6.3 h. These aspects should be kept in mind when the CTDP assay is used for the determination of absolute FFA concentrations of diluted solutions, where a large number of scans has to be accumulated. We are currently performing studies under complete exclusion of atmospheric oxygen: these

experiments are expected to provide more detailed information whether the observed time-dependent changes are caused (a) by oxidation or (b) the limited stability of the derivatization products.

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Notes

The authors declare no competing financial interest.

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