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Quantitative analysis of retinol and retinol palmitate in vitamin tablets using ^{1}H-nuclear magnetic resonance spectroscopy

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Abstract

^{1}H-NMR spectrometry was applied to the quantitative analysis of Vitamin A in four different types of vitamin tablets without any chromatographic purification or saponification. The experiment was performed analysing the H-15 resonance, which appears at δ 4.32 for retinol and δ 4.69 for retinol palmitate, well separated from other resonances in the ^{1}H-NMR spectrum. Compounds were quantified using the relative ratio of the integral of the H-15 signal to that of a known amount of internal standard (200 μg/ml), anthracene. In order to evaluate the feasibility of avoiding the saponification of retinol palmitate in the preparation of samples, several solvents such as dimethylsulfoxide, *n*-hexane, methanol, water, and 0.1 M of HCl were tested as possible extraction solvents. Among these, dimethylsulfoxide showed the best yield of retinol palmitate. This method, using dimethylsulfoxide extraction and ^{1}H-NMR, allows rapid and simple quantitation of retinol palmitate in tablets avoiding tedious saponification.

Keywords: Retinol; Retinol palmitate; ^{1}H-NMR spectrometry; Quantitative analysis

1. Introduction

Vitamin A comprises a number of retinoids of very similar structure—including (*Z*)-isomers—found in animal tissues, which possess similar activity. The principal and biologically most active substance is all-(*E*)-retinol (all-(*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraen-1-ol (Fig. 1). The retinoids (Vitamin A and analogues) are essential for growth and survival. For example, they are necessary for normal vision, night vision and have recently been shown to modulate immune function and infectious disease susceptibility. A deficiency of Vitamin A leads to vision defects, including impairment at low light levels, and a drying and degenerative disease of the cornea [1]. However, excessive intake of Vitamin A can lead to toxic effects, including pathological changes in the skin, hair loss, blurred vision, and headaches. The recommended daily intake of Vitamin A intake is 700–900 μg per day [2]. The stability of Vitamin A varies according to the food selected and the way in which it is prepared and/or stored. It is very unstable to ultraviolet (UV) light radiation and to low pH [3]. This causes both isomerisation of all-*trans*-Vitamin A to the less potent *cis*-forms, and de-esterification of retinol esters to the more labile retinol [4].

This makes it very difficult to estimate the total Vitamin A content of products. To date analysis of Vitamin A analogues is most frequently carried out using methods based on HPLC connected with ultraviolet, fluorescence, or diode array detectors [3,5–8]. The analysis of fat-soluble Vitamin A, however, usually includes several time-consuming purification steps. Pharmacopeias and official food analysis methods include a saponification step, which has the function of converting all Vitamin A forms and possible esters to free retinol, basically because the quantitation method em-

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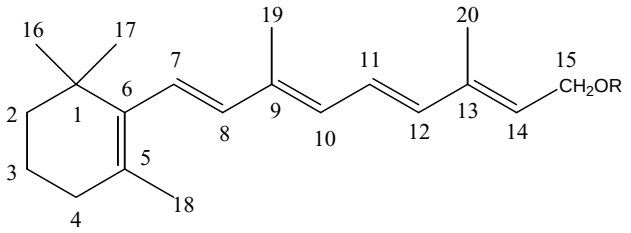
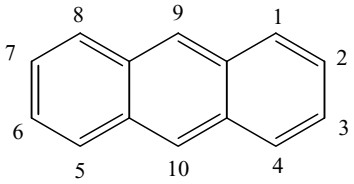


R = H, Retinol
R = $COCH_3$, Retinol acetate
R = Palmitic acid, Retinol palmitate



Anthracene

Fig. 1. Chemical structures of retinol, retinol acetate, and retinol palmitate.

ployed is HPLC or eventually UV spectrophotometry, both of which require the vitamin to be present in a determined form. Additionally, Vitamin A is often extracted together with other lipophilic substances which are part of the sample or, in the case of the tablets, is actually included in a solid often hydrophilic matrix to improve their compressibility. The saponification process eliminates many of these problems, but being a long procedure, with several steps, requires a skilled operator to avoid the loss of Vitamin A due to exposure to light and/or loss due to manipulation. It also means that unstable retinol is analysed instead of the more stable retinol acetate or palmitate which is originally present in the tablets.

The aim of the present study was to develop a direct method for the analysis of Vitamin A in the tablets using ^{1}H-NMR for quantitation of samples obtained without saponification. The use of ^{1}H-nuclear magnetic resonance (NMR) spectroscopy has been developed and applied to the quantitation of natural or synthetic compounds [9,10]. It was done for ginkgolides in *Ginkgo* leaves [11,12], 2,4-dichlorophenoxyacetic acid and sodium 2,2-dichloropropionate [13], and glyphosate and profenofos [14]. These studies show the potential of quantitative NMR spectroscopy as a viable alternative to chromatographic methods. The most outstanding advantages of NMR are the ease of sample preparation, its inherent linearity and the ready assignment of a certain signal to a structure with numerous well-established chemical shifts data bases and spin–spin coupling constants [13].

In this study, ^{1}H-NMR method was evaluated for the analysis of Vitamin A as retinol or retinol palmitate in commercial tablets and compared to a conventional HPLC method. In addition, the results obtained with saponified and non-saponified samples were compared.

2. Experimental

2.1. Materials and reagents

First grade *n*-butanol, dimethylsufoxide, diethylether, ethanol, and *n*-hexane were purchased from Merck Biosolve (Valkenswaard, The Netherlands). HPLC grade *n*-hexane, KOH, Na_2SO_4, pyrogallol, and *di-tert*-butylmethylphenol were from Merck (Darmstadt, Germany). Anthracene, ascorbic acid, retinol, and retinol palmitate were obtained from Sigma (St. Louis, MO, USA) and $CDCl_3$ (99.96%) was from Cortec (Paris, France).

Sample of retinol palmitate in a gelatine matrix (250,000 IU/g, 1 IU = 0.30 μg of retinol or 0.55 μg of retinol palmitate) was used as raw material for the tablets and four types of tablets: Essential Organics Vitamine A® (No. 14412), Essential Organics Vitamine A&D® (No. 14413), Essential Organics Megavites® (No. 14441), Essential Organics All Sports® (No. 14438), were obtained from Tramedico International (Weesp, The Netherlands).

2.2. Extraction

The extraction was performed using three different methods. First the method proposed by Escrivá et al. was used [3]. Twenty tablets of each formulation were powdered and 1.0 g of Essential Organics Vitamine A®, 1.0 g of Essential Organics Vitamine A&D®, 2.0 g of Essential Organics Megavites®, and 5.0 g of Essential Organics All Sports® were weighed, placed in an evaporation flask together with 300 mg of ascorbic acid in 40 ml of ethanol and 10 ml of 50% (v/w) KOH and saponified at 80 °C for 30 min under nitrogen. The resulting solution was transferred to a separating funnel and 50 ml of cold water were added and then extracted with diethylether (4 × 50 ml). The organic extracts were combined and washed with distilled water (4 × 50 ml), and the resulting diethylether phase was dehydrated with anhydrous Na_2SO_4, filtered, and taken to dryness in a rotary evaporator. The residue was dissolved in 1 ml of $CDCl_3$ and 200 μg of anthracene were added by weighing 283 mg of a stock solution of internal standard, which was prepared by accurately weighing 100 mg of anthracene and taking it to volume with $CHCl_3$ in an 100 ml volumetric flask.

As a second extraction method, the official method of the Food and Consumer Product Safety Authority in the Netherlands was used. The same amounts of sample to that of the first method were saponified in 50 ml of ethanol and 5.0 ml of 60% KOH (v/w) at 80 °C for 30 min, together with 1 g of pyrogallol under nitrogen. The resulting solution was extracted with diethyl ether (2 × 100 ml) after adding 100 ml of cold water. The diethyl ether phase was rinsed with water (4 × 100 ml) and 100 mg of *di-tert*-butylmethylphenol were then added. The extract was dried in rotary evaporator. Two hundred microgram of anthracene was added to the residue that was dissolved in 1 ml of $CDCl_3$.

For the extraction of retinol palmitate without saponification, the same amount of each sample was dissolved in 40 ml of dimethylsulfoxide with 300 mg of ascorbic acid and extracted at 80 °C for 30 min. This solution was partitioned with *n*-hexane (4 × 50 ml) and the hexane layers were combined and washed with distilled water (2 × 50 ml) to remove the residual dimethylsulfoxide. The extract was dried with anhydrous Na_2SO_4, filtered, and evaporated in a rotary evaporator. The residue was dissolved in 1 ml of $CDCl_3$ and 200 μg of anthracene were added.

2.3. HPLC analysis

The HPLC instrument consisted of a Waters 626 pump, a 2996 photodiode array detector fixed at 325 nm, and a 717 plus autosampler (Waters, Milford, MA, USA). The sample solution was injected through a 20 μl loop. A LichrosorbSi60 (250 mm × 4.0 mm, S-5 μm) (Merck, Darmstadt, Germany) with a LiChrosorbSi-60 precolumn (10 mm × 4.0 mm S-5 μm) guard column (Merck) were eluted isocratically with the mixture of *n*-hexane:*n*-butanol (98:2). The flow rate was 1.0 ml/min.

2.4. ^{1}H-NMR apparatus and parameters

^{1}H-NMR spectra were recorded in $CDCl_3$ using a Bruker AV 400 spectrometer (400 MHz) equipped with an Indy Silicon graphics computer). For each sample, 256 scans were recorded with the following parameters: 0.13 Hz per point, pulse width (PW) = 30° pulse (6.0 μs), acquisition time (AQ) = 3.97 μs and relaxation delay (RD) = 1.0 s. Free induction decay (FIDs) were processed with LB = 0.3 Hz line broadening prior to Fourier transformation. Phase and base line were corrected manually. For quantitative analysis, peak integral was used.

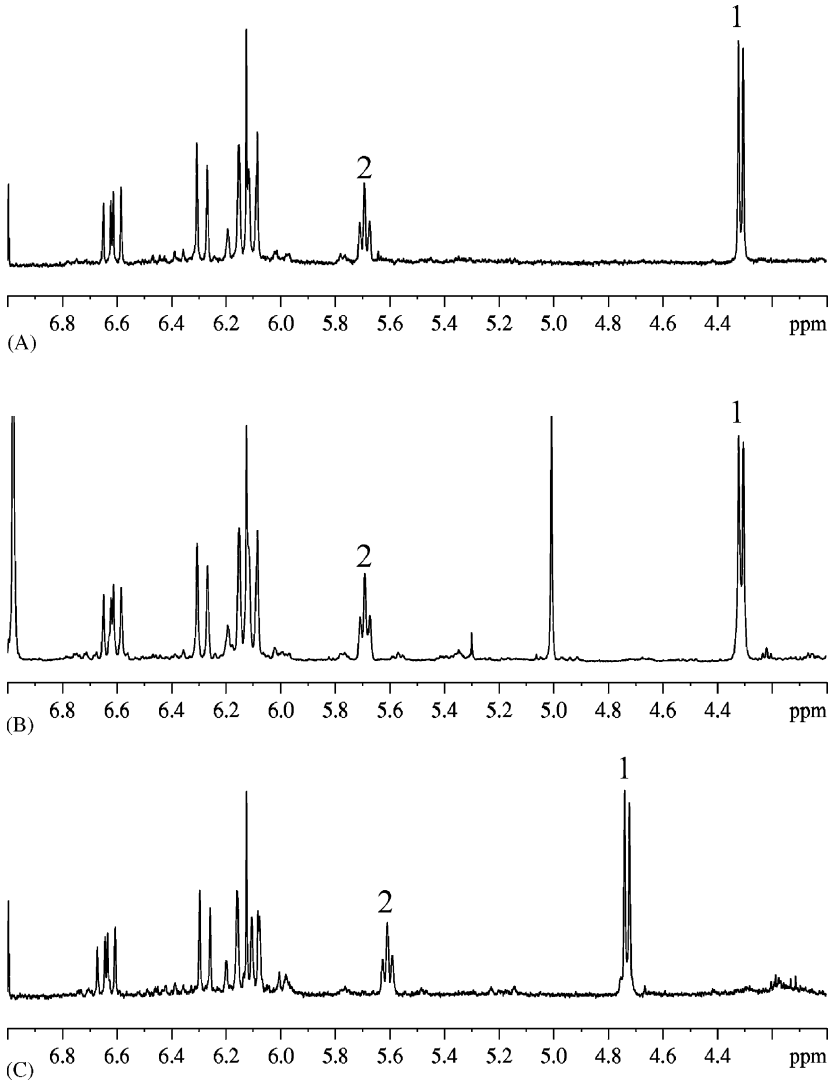


Fig. 2. The 400 MHz ^{1}H-NMR spectrum of the reference compounds: (A) retinol, (B) retinol palmitate after saponification, and (C) retinol palmitate in the range of δ 4.0–7.0 in $CDCl_3$. (1) H-15; (2) H-14.

3. Results and discussion

The ^{1}H-NMR quantitation of Vitamin A as retinol or retinol palmitate in vitamin tablets requires that a target peak is chosen for the analysis. The ^{1}H-NMR spectrum of retinol shows the signals due to five methyl groups at δ 1.03 (6H, s, H-16 and H-17), δ 1.71 (3H, s, H-18), δ 1.89 (3H, s, H-19), and δ 2.00 (3H, s, H-20), six methylenic protons at δ 1.46 (2H, m, H-2), δ 1.61 (2H, m, H-3), and δ 2.02 (2H, t, $J = 6.1$ Hz, H-4), six olefinic protons at 5.69 (1H, t, $J = 7.0$ Hz), d 6.09 (1H, d, $J = 11.2$ Hz, H-10), δ 6.10 (1H, d, $J = 14.1$ Hz, H-8), δ 6.14 (1H, d, $J = 13.9$ Hz), δ 6.28 (1H, d, $J = 15.1$ Hz, H-12), and δ 6.64 (1H, dd, $J = 15.1$ Hz, 11.3 Hz, H-11), and two methylenic protons attached to oxygen at δ 4.32 (2H, d, $J = 7.0$ Hz). Fig. 2 shows that peaks of the two methylenic H-15 protons and one H-14 olefinic proton of retinol and retinol palmitate are detected in a non-crowded region, well separated from each other. The H-15 peak of retinol is detected at δ 4.32 (2H, d, $J = 7.0$ Hz) but that of retinol palmitate is shifted to δ 4.69 (2H, d, $J = 7.2$ Hz). The H-14 is slightly shifted to upfield, from δ 5.69 (1H, t, $J = 7.0$ Hz) to 5.53 (1H, t, $J = 6.9$ Hz). H-15 is the doublet of two protons and H-14 is a triplet of a single proton. Therefore, the H-15 signal is consequently considerably higher than H-14 signal. The easy recognition of the chemical shifts of retinol and retinol palmitate, the detection in a less crowded region, and a simple splitting pattern resulting in a higher intensity, makes H-15 the preferable target signal for the analysis in ^{1}H-NMR.

As a next step, different compounds have to be evaluated as possible internal standards since quantitative NMR generally requires addition of an internal standard in order to calculate the amount of analyte present in the sample by comparison of the integral ratios. The ^{1}H-NMR signals of anthracene do not overlap with sample peaks and particularly show a sharp singlet at δ 8.43 (H-9 and H-10) making it a good choice for this case.

Among the ^{1}H-NMR acquisition parameters, determination of relaxation time T1, value is the most important factor

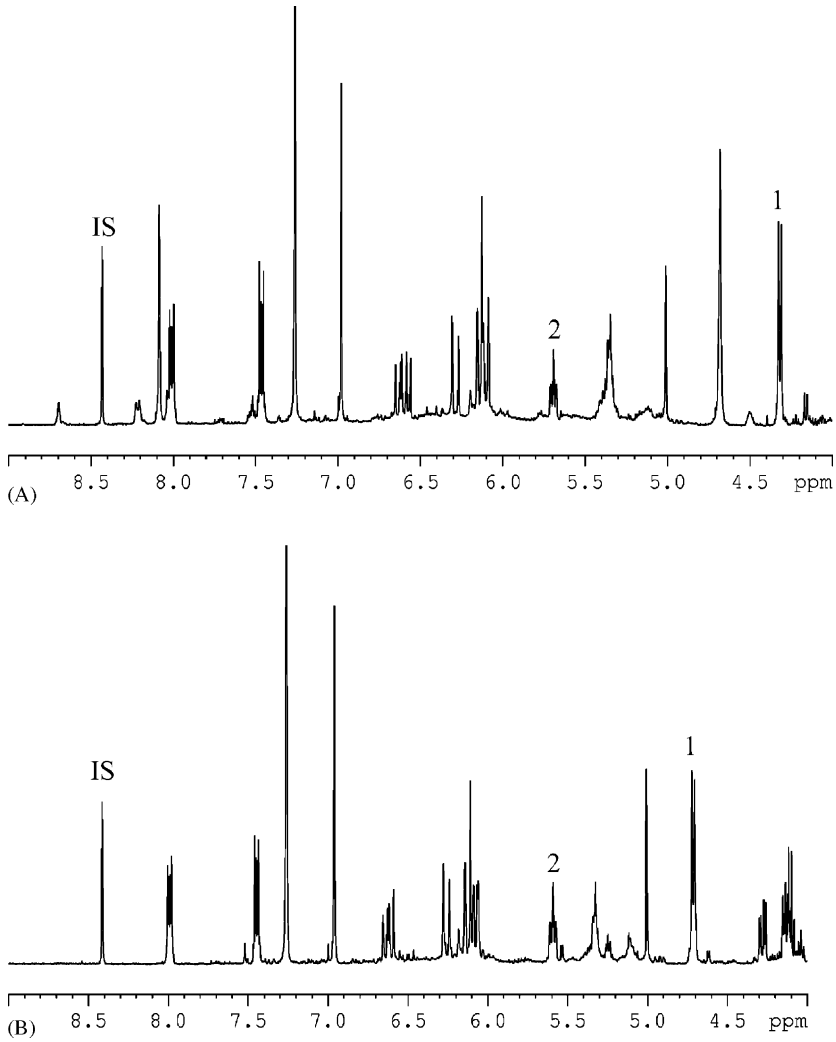


Fig. 3. The 400 MHz ^{1}H-NMR spectrum of: (A) retinol after saponification of retinol palmitate raw material and (B) retinol palmitate raw material without saponification in the range of δ 4.0–7.0 ($CDCl_3$). (1) H-15; (2) H-14; IS: H-9 and H-10 of anthracene (internal standard).

because insufficient relaxation results in underestimation of the amount present in the sample. For example, the relaxation time for nitroaromatic compounds has to be prolonged because of their slow relaxation protons [15]. The relative integral of anthracene protons on each relaxation delay (5, 10, 35 s) were investigated. The integration using 35 s was found to be increased by only 4% compared to using 5 s. However, the running time of ^{1}H-NMR with 35 s was four times higher than that 5 s. Therefore, 5 s was chosen as relation time. For the confirmation of the relaxation time used in this study, three different solutions containing a known amount of retinol palmitate were analysed and the results were compared with the real amount of the compound. The error range was below 5%.

The amount of Vitamin A was calculated using the following equation:

$$\text{Quantity (mg, } 1\,\text{IU} = 0.3\,\mu\text{g of retinol or } 0.55\,\mu\text{g of retinol palmitate)} = \frac{\text{integral (VA)}}{\text{integral (IS)}} \times \frac{\text{MW (A)}}{89.1} \times \text{weight (IS)}$$

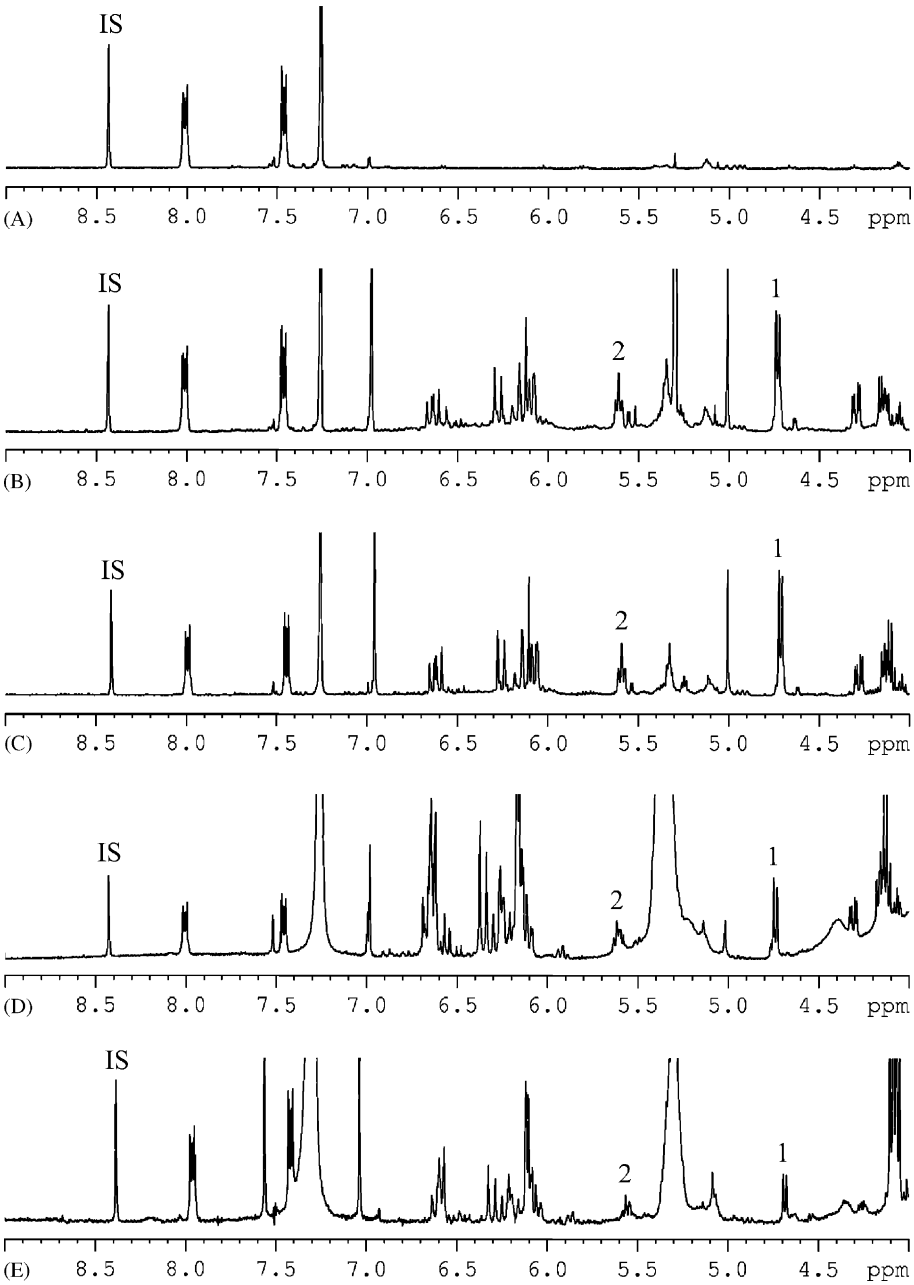


Fig. 4. The 400 MHz ^{1}H-NMR spectrum of: (A) blank sample, (B) Essential Vitamine A®, (C) Essential Vitamine A&D®, (D) Essential Organics Megavites®, and (E) Essential Organics All Sports® in the range of δ 4.0–7.0 ($CDCl_3$). (1) H-15; (2) H-14; IS: H-9 and H-10 of anthracene (internal standard).

Table 1
The contents of Vitamin A in vitamin tablets analysed by ^{1}H-NMR spectroscopy (calculated as mg of retinol/g of tablets ± S.D.)

Sample	^{1}H-NMR		HPLC
	Saponification	Non-saponification	Saponification
Essential Vitamine A®	1.00 (±0.05)	1.09 (±0.11)	0.98
Essential Vitamine A &D®	1.62 (±0.12)	1.37 (±0.24)	1.34
Essential Organics Megavites®	0.48 (±0.08)	0.48 (±0.06)	0.57
Essential Organics All Sports®	0.15 (±0.04)	0.15 (±0.02)	0.15

All results were based on five experiments.

where integral (VA) = the peak area of the H-15 of retinol or retinol palmitate; integral (IS) = the peak area of the H-9 and H-10 of anthracene; MW (A) = the molecular weight of retinol or retinol palmitate divided by 2 because there are two H-15 protons (143.2 for retinol and 262.6 for retinol palmitate); weight (IS) = the amount of anthracene added (200 μg in this report); the factor of 89.1 is obtained from the molecular weight of anthracene divided by 2 (two protons such as H-9 and H-10).

The retinoids are sensitive to oxidation, so antioxidants such as pyrogallol, *di-tert*-butylmethylphenol, and ascorbic acid have been used during extraction or saponification [3,16]. One of the samples, Essential Vitamine A® was analysed by two saponification methods of which one used pyrogallol as a water soluble antioxidant and *di-tert*-butylmethylphenol for the organic solvent, and the other used only ascorbic acid. Both of methods showed similar results, 1.04 (±0.11) and 1.00 (±0.05) mg/g (calculated as the amount of retinol), respectively. Thus, the simpler antioxidant, ascorbic acid was selected for the analysis of Vitamin A in tablets.

Saponification is an extremely tedious process and factors such as sample size, potassium hydroxide concentration, temperature and time, and sample type [16] have to be optimised. The convenience of avoiding this time-consuming step led us to evaluate the feasibility of the analysis of retinol palmitate without saponification using ^{1}H-NMR. Dimethylsulfoxide extraction followed by partitioning into *n*-hexane

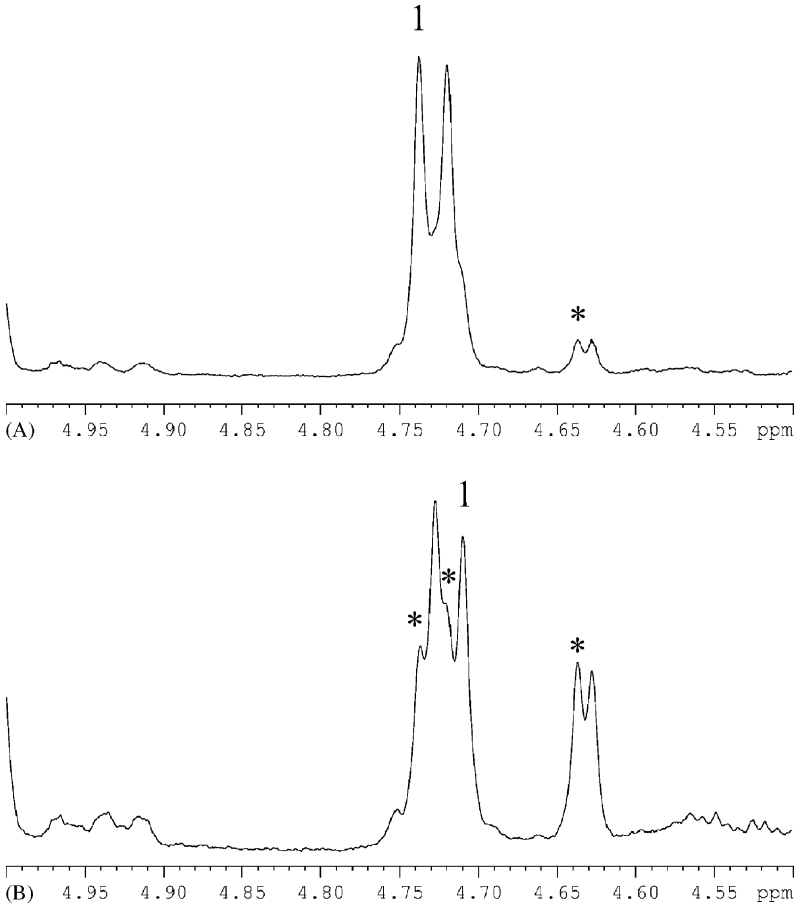


Fig. 5. The 400 MHz ^{1}H-NMR spectrum of Essential Vitamine A®: (A) after 10 min and (B) 24 h in the light in the range of δ 4.5–5.0 ($CDCl_3$). (1) H-15; (*) possible isomerised peaks of retinol palmitate.

extraction showed best yields of retinol palmitate among the extraction solvents evaluated in this study (chloroform, ethanol, *n*-hexane, methanol, water, and 0.1 M HCl). Pure retinol palmitate dissolves easily in lipophilic solvents such as *n*-hexane or chloroform, but if it is bound to a matrix such as gelatine, it cannot be extracted with these solvents. Fig. 3 shows the results obtained from dimethylsulfoxide extraction of the retinol palmitate raw material in which oily retinol palmitate is bound to a hydrophilic matrix to achieve a good formulation for the tablets. The recoveries of the compound by saponification and direct extraction were found to be 90.5 and 90.9%, respectively.

Four types of vitamin tablets were analysed using dimethylsulfoxide extraction and ^{1}H-NMR spectroscopy. Fig. 4 shows the ^{1}H-NMR spectrum of the tablet extracts, together with that of a blank sample which was processed only with ascorbic acid. There is no interferencing signal from ascorbic acid in the blank sample. All ^{1}H-NMR spectra of vitamin tablet extracts show a clear separation of the H-15 and H-14 signal of retinol palmitate. Essential Organics Vitamine A® and Essential Organics Vitamine A&D® contain predominantly Vitamin A. However, other tablets, Essential Organics Megavites® and Essential Organics All Sports® contain most water and oil soluble vitamins and β-carotene. Even for these complex samples, the target signal is well separated. The amounts of retinol palmitate in the tablets evaluated using dimethylsulfoxide extraction and ^{1}H-NMR spectroscopy are listed in Table 1 and compared with those obtained from saponification method combined with ^{1}H-NMR or conventional HPLC method. The analytical results obtained by the non-saponification method with ^{1}H-NMR show similar results to those of saponification and HPLC. It indicates that the quantitative ^{1}H-NMR method combined with dimethylsulfoxide extraction can be a good alternative a conventional HPLC method.

Finally, the detection limit of retinoids in ^{1}H-NMR spectroscopy was measured and found to be 0.1 μg/ml with 256 scan numbers. It is quite high compared to that achieved with HPLC–UV. However, considering that the Vitamin A content in tablets is high (e.g. more than 500 μg per tablet) this low sensitivity does not constitute a drawback for its applicability. Furthermore, the method allows for concentration of the Vitamin A content during the extraction process if necessary.

In addition to the quantitation, importantly ^{1}H-NMR can also be used qualitatively for the profiling of retinoids which can be isomerized relatively easily under various conditions such as under ultraviolet light (UV) radiation or low pH. The configurational changes of retinoids, e.g., 9- and 13-*cis*-isomerization, shift the resonance of H-15 slightly downfield [17], making it easy to detect. An example of this evaluation can be shown in Fig. 5. The isomerization of retinol palmitate is clearly detected in the ^{1}H-NMR spectrum.

4. Conclusion

This method provides a simple, accurate and precise solution for the analysis of Vitamin A in tablets containing other fat and water soluble vitamins, plus additional nutrients, using ^{1}H-NMR spectrometry without tedious sample treatment procedures such as saponification. It was shown to be valid for analysing Vitamin A and/or its esters, with a very simple sample extraction process, the use of ^{1}H-NMR allows the measurement of free retinol and its esters, even in the presence of other compounds that could interfere in its quantitation, for example by HPLC. This method could thus the complementary with HPLC, particularly avoiding the need for elaborate saponification methods, and measuring the actual content of the preparation.

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