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Spectral Properties of Oxidation Products of Retinyl Acetate

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Introduction

It is known that retinol (vitamin A_1) and its esters, as commonly used for the enrichment of certain selected foods with vitamin A, suffer destruction during technological treatment and storage. In this connection, the character of products obtained by the effect of irradiation has been studied (1–6). Also products resulting from retinol and retinyl acetate in the solutions containing water and alcohols have been analyzed especially by Japanese authors with the aim of ascertaining the stabilities of drug forms (7, 8). However, little information is available about the properties of substances resulting in oxidation processes. Thus some closer characterization of the oxidation products is the subject of the present communication.

Owing to their structure (4–6 conjugated double bonds), the oxidation products show spectral properties similar to those used as a basis for the determination of vitamin A. They absorb radiation in the ultraviolet region in some part of the 270–390 nm range. It might therefore be anticipated that the absorption of the artefacts at 325 nm will not be negligible (9). The same applies to the fluorescence spectra with the exception of anhydroretinol. The oxidation products are excitated by radiation in the 330 nm range. Although their emission maxima are shifted to lower wavelengths, they nevertheless emit radiation in the range near 500 nm, i. e. in the analytical range of retinol and retinyl acetate. The oxidation products of retinyl acetate form complex compounds with Lewis acids as well as the active substance. Although both the light absorption and intensity of these complexes differ from those of standard retinol or retinyl acetate, interference will occur also in the colorimetric method (10) and its recently reviewed modifications (11). In our experimental work we used as a model substance the standard oily preparation of retinyl acetate that has been applied for the enrichment of foods with vitamin A.

Oxidation was carried out by accelerated lipid oxidation test – the Schaal Method. Fourteen substances were isolated by preparative layer chromatography (12). There are outlined the spectral characteristics of the substances obtained.

Materials and Methods

Chemicals

Retinyl acetate oily preparations (Alc Société de chimie organique et biologique, Commentry, 50%).

Solvents reagent grade or for UV spectrometry, silica gel HR (Merck, Darmstadt). Carr-Price reagent 22 g $SbCl_3$ + 100 g $CHCl_3$.

Apparatus

Double beam recording spectrophotometer for ultraviolet, visible and near infrared region SP-700 (Pye-Unicam, Cambridge). Fluorescence spectrophotometer Perkin-Elmer MPF 4 (Norwalk, Connecticut). Universal UV lamp 254, 366 nm (Camag, Muttenz).

Methods

Retinyl acetate was oxidized in 1 cm layer in the dark at 60 °C with free access of air for 25—40 days until reduction of retinyl acetate concentration to 5–10% of the original value. The artefacts were isolated from the oxidized retinyl acetate by preparative layer chromatography (plates with 3 mm silica gel HR, hexane-diethyl ether as mobile phase, ratios 95:5 to 10:90, detection 366 nm). Pure substances were obtained by 3 to 5-fold development as described in detail in another communication (12).

Results and Discussion

Table 1 shows the course of oxidation of the standard retinyl acetate preparation. The process was followed by UV spectra (absorptivity at 325 nm in ethanol) (9), colorimetry with $SbCl_3$ (10) and fluorometry (13). The corrected UV values in table 1 were obtained by application of the rule of absorbance additivities of retinyl acetate and the oxidation products. The absorptivities of the oxidation products (ε_p) were determined after their chromatographic isolation. The mean values were 0.033 and 0.010 $cm^2\ \mu g^{-1}$ at 285 and 325 nm, respectively. The absorptivities of retinylacetate (ε_r) at 285 and 325 nm were 0.0461 and 0.1561 $cm^2\ \mu g^{-1}$, respectively.

Solution of the equations:

$$A_{325} = c_r \cdot d \cdot \varepsilon_{r325} + c_p \cdot d \cdot \varepsilon_{p325}$$
$$A_{285} = c_r \cdot d \cdot \varepsilon_{r285} + c_p \cdot d \cdot \varepsilon_{p285}$$

yielded the relations for calculation of the corrected UV values:

$$7.04 \cdot A_{325} - 2.13 \cdot A_{285} = c_r\ (\mu g/ml)$$
$$33.2 \cdot A_{285} - 9.84 \cdot A_{325} = c_p\ (\mu g/ml)$$

Table 1. Oxidation of retinyl acetate

Time (days)	% of retinyl acetate determined by			
	UV spectra (9)	UV spectra corr.*	Colorimetry (10)	Fluorometry (13)
0	47.2	48.0	48.2	47.0
1	42.5	43.2	43.8	42.0
3	32.5	31.8	29.0	28.3
4	32.1	30.4	33.6	30.6
5	29.0	28.7	32.2	26.4
7	29.2	28.5	32.2	26.4
10	25.3	23.4	25.4	21.5
12	20.5	19.0	21.5	18.7
14	18.9	17.5	20.9	16.2
18	16.8	15.5	18.0	13.5
19	14.7	13.2	16.6	11.8
21	12.7	10.9	14.3	11.5
24	11.8	9.7	13.9	9.6
26	11.6	9.4	11.8	8.2
28	10.6	8.5	10.2	8.2
31	10.3	7.7	9.7	7.9
34	8.5	6.3	9.5	6.5
36	8.2	6.1	7.9	5.5
40	6.7	3.9	5.8	3.7

* Corrected for the presence of oxidation products as described in the text.

(A_{325} and A_{285} are the absorbances at the given wavelenghts, d = cell thickness = 1 cm, c_r and c_p the concentrations of retinyl acetate and oxidation products in μg/ml.)

Table 1 shows to what extent the analytical procedures are affected by the rise of oxidation product concentration. The highest interfering effect was observed when applying the Carr-Price Method and uncorrected UV spectrometry, while the lowest effect occurred in fluorometry, where no apparent interference was observed in an oxidized sample in which the amount of oxidation products was 90%. The corrected ultraviolet data approximated to those of the fluorometric range.

The actual concentration of nonoxidized retinyl acetate in the sample at day 40 of exposure to oxidation was determined also by chromatographic isolation of the substances (12). The value of 3.3%, as was thus ascertained, differs least from those of fluorometry (3.7%) and corrected UV spectra (3.9%).

Nevertheless, table 1 shows the effect of the oxidation products upon determination of retinyl acetate only summarily. In order to evaluate the influence of the individual products, it was necessary to obtain them in a pure state. The preparative layer chromatography (12) was well suited to serve this purpose, allowing

to obtain fourteen substances. Table 2 shows their weight distribution in oxidized retinyl acetate and compiles their spectral characteristics. Figure 1 shows the UV and the visible spectra with $SbCl_3$ and figure 2 the excitation and emission fluorescence spectra.

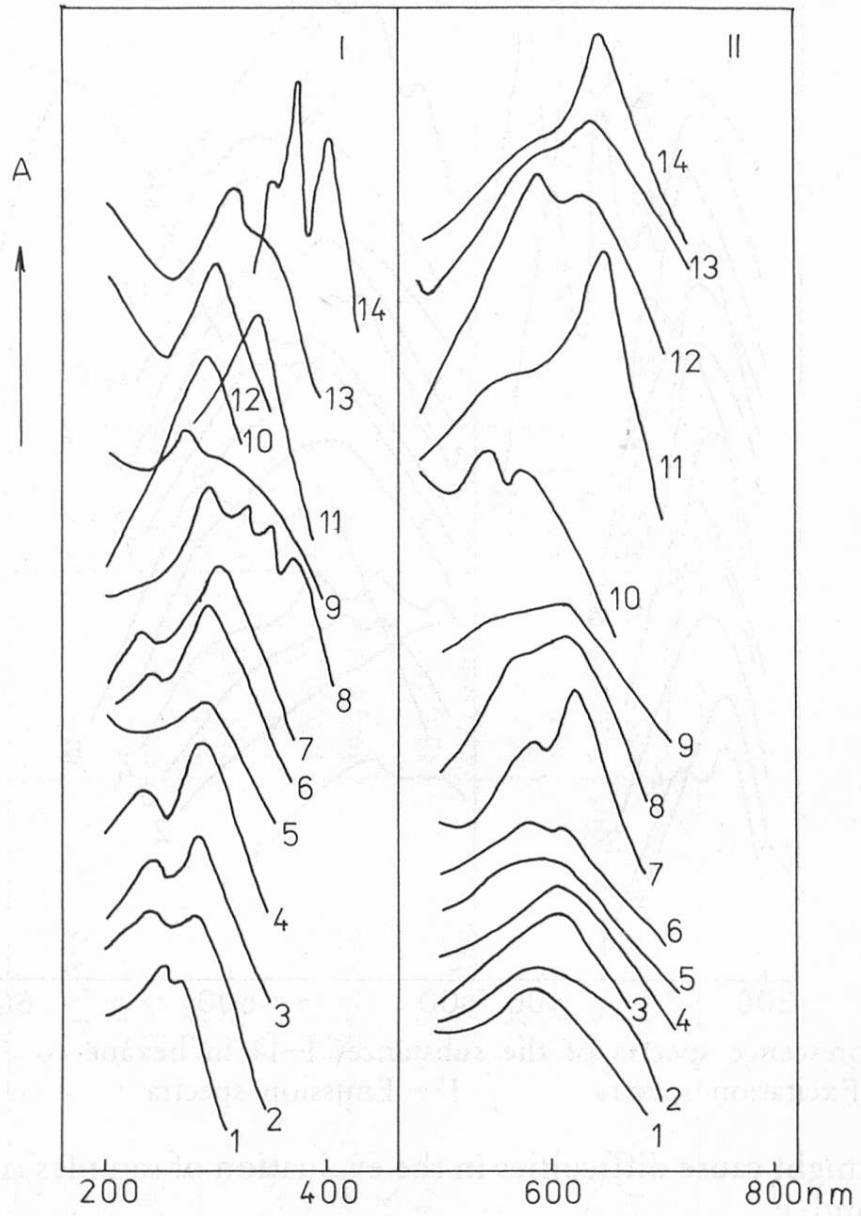

Fig. 1. Absorption spectra of the substances 1—14
I — Ultraviolet spectra in hexane
II — Visible spectra of complexes with $SbCl_3$ measured after 1 minute

The polar substances all exhibit an absorption maximum in the 270—285 nm range. Some of them are characterized by a second band with a maximum in the 240—250 nm range. The shift to lower wavelengths in comparison with retinol or retinyl acetate indicates that the system of conjugated double bonds has shortened due to the addition of oxygen on double bonds and the formation of compounds with oxigenated moieties. The absorption is lower and comparable in its entity and spectral range with those of tocopherol and tocopheryl acetate,

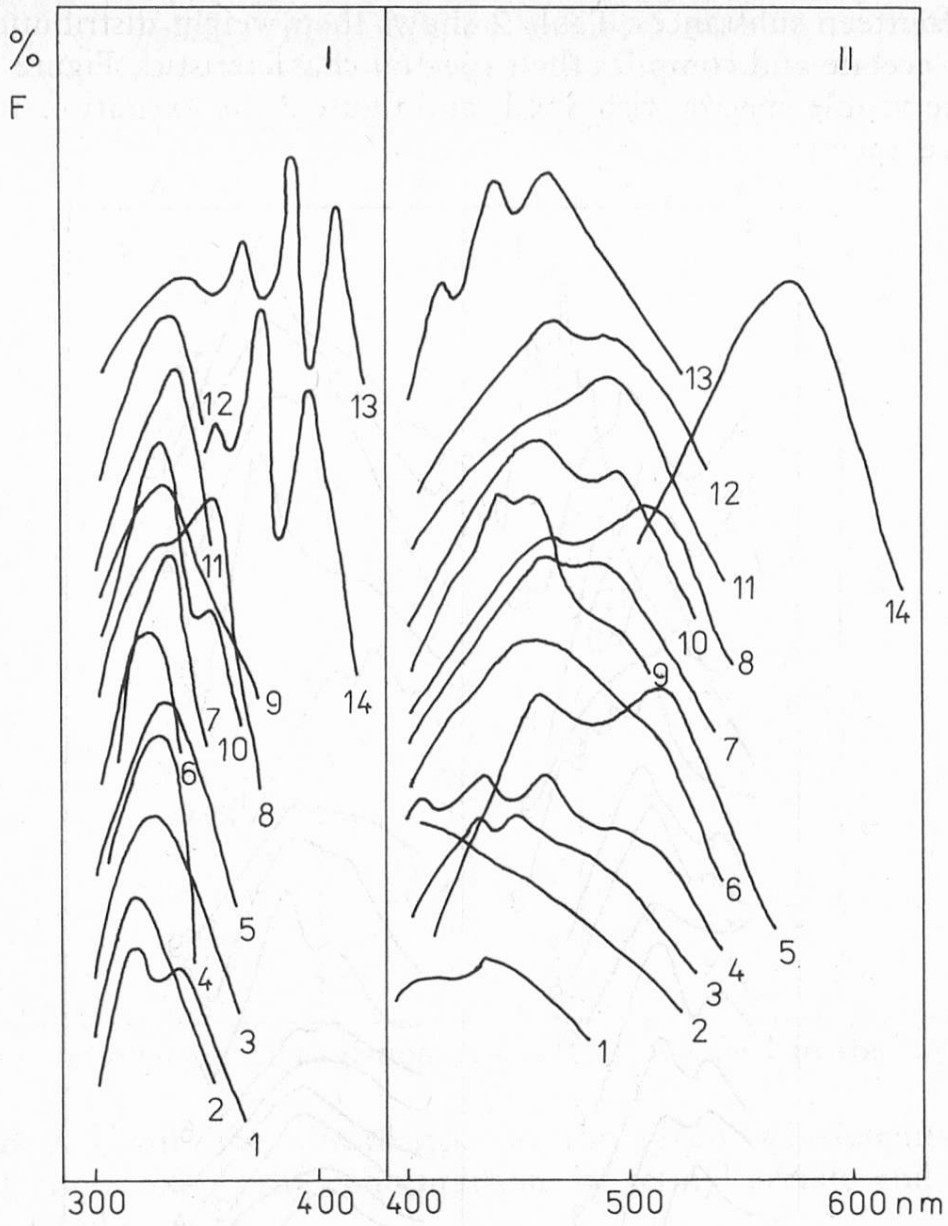

Fig. 2. Fluorescence spectra of the substances 1—14 in hexane
I — Excitation spectra II — Emission spectra

which also might cause difficulties in the evaluation of samples containing the vitamins A and E.

The less polar compounds (substances 8, 14 — table 2) are characterized by multiplets of well resolved peaks shifted to higher wavelengths in comparison with retinyl acetate, which again proves changes in the system of conjugated double bonds caused either by isomeration (e. g. retro) or dehydration (e. g. anhydro and isoanhydro structures). The less polar substances exhibit a strong ultraviolet absorption, but their interference is less significant as they occur only in low concentrations (table 2).

As for the fluorescence spectra, the excitation maxima of the polar substances lie at higher wavelengths than might be expected from the ultraviolet absorption peaks, i. e. in the 310—330 nm range. Nevertheless, the emission maxima are shifted to lower wavelenghts than that of retinyl acetate (490 nm in the uncorrected

Table 2. Properties of the fractions obtained by preparative layer chromatography (12)

Substance No.	Concentration in oxidized retinyl acetate %	UV spectra		Fluorometry		Complex with $SbCl_3$			
		max. nm	absorbance ratio*	excitation max. nm	emission max. nm	after 1 min		after 5 min	
						max. nm	colour	max. nm	colour
1	0.9	262, 272	1:0.953	315, 335	435	585	violet	—	grey
2	10.1	242, 278	1:0.965	315	0	588	violet	570, 472	pink-brown
3	26.2	242, 283	0.965:1	325	430, 445	605	blue	548, 470	red-brown
4	3.5	240, 285	0.968:1	330	405, 432, 458	605	blue	540, 470	red-brown
5	3.3	285	—	332	455, 510	605	violet-blue	545	pink
6	15.8	242, 285	0.882:1	320	450	575–605	violet-blue	575, 470	red-brown
7	3.5	237, 298	0.800:1	332	460, 485	620, 585	blue	570, 480, 620s	violet-red
8	0.9	290, 328, 350, 370	1:0.905:0.778: 0.495	330, 350	465, 510	608	blue	—	violet-grey
9	1.8	270, 285s	1:0.945	332	435, 458	608	violet-blue	—	pink-grey
10	2.6	285	—	330, 350	460, 490s	540, 565s	violet-red	540	red
11	29.0**	325	—	332	490	625	blue	625, 565	violet-blue
12	0.6	292	—	330	462, 500	585, 615s	violet-blue	575, 615s	violet
13	0.8	313, 325s	1:0.738	330, 365, 382, 405	412, 435, 460	625	blue	625, 580s	violet-blue
14	1.0	351,371,392	0.690:1:0.870	355, 372, 394	568	628	blue	628, 570s	violet-blue

Note: UV and fluorescence spectra were measured in hexane, complexes with $SbCl_3$ in chloroform solutions
* Relative intensities of the UV absorption maxima, the highest peak intensity = 1
s = shoulder
** Nonoxidized retinyl acetate migrating with the residue of oil from the standard preparation

spectrum in hexane). The emission peaks are broad, and there always occurs an essential amount of the total radiation emitted at 490 nm. The low interference (table 1) can be explained by a much lower total emission in comparison with retinyl acetate. Fine structure is observed especially in the excitation spectra of the nonpolar substances, which is again in agreement with the features of ultraviolet absorption spectra. This is typical especially for anhydroretinol (substance 14).

The spectra of complexes: oxidation product — $SbCl_3$ were measured in $CHCl_3$ after 1 and 5 min after the reagent has been added. The changes in both the colour intensity and region were not so quick as after 6 seconds (10), which allowed the spectra to be scanned in the whole 400—650 nm range. All tested substances give colour compounds with $SbCl_3$. There result broad peaks with absorption in the whole 400—650 nm range. The intensity of absorption increases at lower wavelenghts and decreases at higher ones with time. The ratio of intensities of different peaks causes changes in colour — they are also given in table 2. The absorption at 620 nm always equals a significant percentage of that at the measured maximum, and therefore interference might be expected even in cases when the colour of the complex is not blue. The spectra of the complexes can also be used for the characterization of the substances; they are typical in position, intensity and the time dependence of intensity.

The results outlined indicate that the oxidation products interfere with all conventional spectral procedures for retinyl acetate or retinol determination. They cause a positive error, the extent of which depends on the spectral character of the products involved.

Fluorometric determination seems to be utmost promising for the evaluation of samples containing the oxidation products, owing to the fact that their effect is least pronounced. Further quantitative evaluation is necessary and will be reported elsewhere.

Chromatographic techniques can be used as a preliminary procedure before the spectral evaluation, but only in some single cases. The data concerning the spectral properties of retinyl acetate oxidation products might also be applied in HPLC analyses of oxidized samples.

Summary

Fourteen substances chromatographically isolated from oxidized retinyl acetate are characterized by their spectral properties (fluorescence and ultraviolet spectra, spectra of complex compounds with $SbCl_3$). The course of oxidation was followed by ultraviolet and visible spectrometry and fluorometry.

Zusammenfassung

Vierzehn mittels präparativer Schichtchromatographie aus oxidiertem Retinylacetat isolierte Substanzen wurden aufgrund ihrer spektralen Eigenschaften (Ultraviolett- und Fluoreszenzspektren, Spektren der Komplexe mit $SbCl_3$) charakterisiert. Der Oxidationsverlauf wurde mittels Spektrometrie im UV- und im sichtbaren Bereich sowie mittels Fluorometrie verfolgt.

Résumé

Quatorze substances, isolées par chromatographie sur plaques à l'échelle préparative à partir d'acétate de rétinyle oxidé, ont été caractérisées sur la base de leurs propriétés spectrales (spectres ultraviolets, spectres des complexes à $SbCl_3$ et spectres de fluorescence). Le cours de l'oxydation est suivi par spectrométrie dans les régions ultraviolette et visible ainsi que par fluorométrie.

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