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Separation of geometrical retinol isomers in food samples by using narrow-bore high-performance liquid chromatography

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Abstract

By using an analytical column of 100 mm × 2 mm I.D. (narrow-bore) with a 3-μm stationary phase and a mobile phase consisting of 1-octanol in *n*-hexane as modifier, seven geometrical isomers of retinol were separated. In the unsaponifiable matter of food samples, six retinol isomers were identified. Heat and microwave treatment of foods such as milk and liver induced the formation of *cis*-isomers of retinol.

1. Introduction

HPLC is the most commonly applied method in retinol analysis. In most cases reversed-phase HPLC is used as a rapid method for the determination of total retinol contents in food samples [1–5]. Retention times are short and reproducible. By using reversed-phase material as the stationary phase and mixtures of methanol and water as the mobile phase in HPLC, the geometrical isomers of retinol are not separated completely. The separation of only 13-*cis*- and all-*trans*-retinol has been described [6]. It is presumed that isomers other than 13-*cis*- and all-*trans*-retinol co-elute with one of them. By using reversed-phase HPLC, the different specific extinctions of the isomers cannot be differentiated. *cis*-Isomers mostly absorb UV radiation less intensively than does the all-*trans*-isomer. Hence the determination of total retinol may lead to underestimation of the retinol content if *cis*-isomers are present [7]. Biological vitamin A activities differ between the isomers. All-*trans*-retinol is defined to possess 100% vitamin A activity. The activities of the *cis*-isomers range from 15% for 11,13-di-*cis*-retinol to 75% for 13-*cis*-retinol [8].

An exact determination and calculation of the vitamin A activity in a food sample is possible only with a knowledge of the isomer distribution.

Several laboratories have reported the HPLC separation of retinol isomers. Some workers only focused on the separation of the main isomers all-*trans*- and 13-*cis*-retinol [9,10]. Others did not achieve a baseline separation of the minor components eluting at very similar retention times to the main isomers [11,12].

Stancher and Zonta separated isomers of retinal, retinol (vitamin A_1) and 3-dehydroretinol (vitamin A_2) in standard solutions [13] and also

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in extracts of the unsaponifiable matter of fish samples [14,15]. They applied a normal-phase HPLC column of standard size. As mobile phases they used *n*-hexane with 1-octanol or 2-propanol as modifier.

In food samples other than fish, no data on the exact retinol isomer distribution are available. They do not contain 3-dehydroretinol (vitamin A_2) in detectable concentrations. Hence most attention should be devoted to the separation of retinol (vitamin A_1) isomers. Also, the effect of heat treatment of fermentation on the retinol isomer pattern in food samples has not been studied so far. Therefore, commercially processed foods such as liver sausage or infant food containing liver in preservation vessels were taken for isomer determination. We employed a narrow-bore HPLC column of I.D. 2 mm (in contrast to 5 mm for standard columns) filled with spherical silica gel as stationary phase. The length (100 mm) was also shorter than usual. By using narrow-bore columns it was possible to reduce the flow-rate, which led to a decrease in solvent usage in comparison with the application of standard columns. Good separation results were obtained because of the small particle size (3 μm) while having short retention times.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a pump (Model 5000; Varian, Palo Alto, CA, USA), a 10-μl loop injector (Rheodyne, Cotati, CA, USA), a photodiode-array detector (Series 991; Waters–Millipore, Milford, MA, USA) with an 8-μl flow cell and a computerized evaluation system (Waters 990 Series PDA, version 6.22 software).

A guard column (7 mm × 2 mm I.D.) was connected to the analytical column (100 mm × 2 mm I.D.) (Knauer, Berlin, Germany), both filled with 3-μm Spherisorb SW silica gel (Phase Separations, Queensferry, Clwyd, UK).

2.2. Reagents and materials

The standards used were all-*trans*-retinol 13-*cis*-retinol and all-*trans*-retinol palmitate (all from Sigma, St. Louis, MO, USA).

The solvents used were absolute ethanol, light petroleum (Merck, Darmstadt, Germany), HPLC-grade *n*-hexane (Merck) and 1-octanol (Sigma). Other chemicals and reagents used were potassium hydroxide pellets, ascorbic acid, Tris [tris(hydroxymethyl)aminomethane] crystalline iodine and Kieselguhr Extrelut (all from Merck), BHT (*tert.*-butylhydroxytoluene) (Fluka, Buchs, Switzerland) and steapsine (crude porcine pancreas lipase) (Sigma). Materials used were glassware of light protective, erg. actinic glassware, disposable cartridges (15 cm length, 2.5 cm diameter) for refilling with Kieselguhr.

2.3. Photolysis

Ethanolic solutions with a concentration of 25 μg/ml 13-*cis*- or all-*trans*-retinol or 100 μg/ml all-*trans*-retinyl palmitate were purged with nitrogen to prevent oxidation and exposed to sunlight for 2 h. The yields of the photolytic process were measured by HPLC with photodiode-array detection.

2.4. Reaction with iodine

According to the method of Brown and Wald [16], 2 ml of a 0.4 mg/ml iodine solution in ethanol were added to 3 ml of a 100 μg/ml ethanolic solution of all-*trans*-retinol palmitate. After adding 60 ml of absolute ethanol, the mixture was stirred for 2 min at room temperature and exposed to daylight. The 11-*cis*-isomer of retinol was obtained in considerable amounts by this reaction.

2.5. Enzymatic influence

A 3-ml volume of a 100 μg/ml ethanolic retinol palmitate solution was added to a reaction mixture consisting of 90 ml of absolute ethanol, 0.5 ml of 1% ethanolic BHT solution,

10 ml Tris buffer (0.1 *M* Tris, pH 9.0) and a steapsine suspension of 0.1 g in Tris buffer. Enzymatic hydrolysis was carried out with magnetic stirring for 45 min at room temperature. The enzymatic reaction was stopped by adding 5 ml of 60% aqueous KOH.

2.6. Alkaline saponification procedure

Samples

Amounts of 1.0–10.0 g of the homogenized sample were weighed and the solid sample matrix was stirred with 30 ml of water. Volumes of 40 ml of the fluid samples were pipetted into a 250-ml round-bottomed flask. For saponification, 12 ml of 60% aqueous KOH, 80 ml of absolute ethanol, 0.5 ml of 1% ethanolic BHT solution and 0.5 g of ascorbic acid were added. To prevent oxidation, the reaction flask was purged with nitrogen and then connected to a balloon filled with nitrogen. Digestion was carried out for 16 h at room temperature on a magnetic stirrer.

Standards

Approximately 100 mg of retinyl palmitate were weighed exactly into a 100-ml volumetric flask and dissolved in *n*-hexane. The stock solution was diluted 1:100 with ethanol. Volumes of 5–35 ml of this standard solution were saponified according to the method used for the samples.

Extraction

The saponified solution was rinsed into a 250-ml volumetric flask and diluted to volume with water and absolute ethanol to yield a volumetric ratio of water to ethanol of 1:1. An aliquot of 20 ml was pipetted on to a Kieselguhr-filled disposable cartridge of synthetic material. After 20 min elution was carried out with 50 ml of light petroleum. The collected eluate was evaporated using a rotary evaporator. The remaining solvent was removed by purging with nitrogen. The residue was dissolved in volumes of 2–50 ml of isooctane depending on the presumed vitamin A concentration in the sample.

3. Results and discussion

Samples of untreated and photoisomerized standards were injected into the HPLC system and chromatographed employing mobile phases consisting of *n*-hexane and 0.3% or 0.25% of 1-octanol. A decrease in the percentage of 1-octanol led to longer retention times.

Working with a mobile phase containing only small percentages of modifier led to the problem that the stability of the composition became uncertain. The retention times tended to vary by up to 10% during one working day because of evaporation of *n*-hexane. As the corresponding behaviour of all isomers was identical, relative retention times with respect to all-*trans*-retinol were calculated. Table 1 reports relative retention times of all detectable isomers as averages of five determinations (for 11,13-di-*cis*-retinol only two determinations were available).

Fig. 1 shows chromatograms of photoisomerized standard solutions of 13-*cis*- and all-*trans*-retinol. Only of 13-*cis*- and all-*trans*-retinol pure standards were available.

The peak identification of other isomers was achieved by comparison of the spectra obtained in this work with literature data. Stancher and Zonta [13] also applied this type of peak confirmation. Spectra of 13-*cis*-, 9,13-di-*cis*-, 9-*cis*-

Table 1
Relative retention times of *cis*-retinol isomers with respect to all-*trans*-retinol

Isomer	Relative retention time[a]	R.S.D. (%)[a]
11-*cis*-	0.510	2.4
11,13-Di-*cis*-[b]	0.568	0.5
13-*cis*-	0.672	0.7
9,13-Di-*cis*-	0.740	0.8
9-*cis*-	0.877	0.6
7-*cis*-	0.924	0.5
All-*trans*-	1.000	0.0

Stationary phase, 3-μm silica gel; column, 100 mm × 0.2 mm I.D.; mobile phase, 0.3% 1-octanol in *n*-hexane; flow-rate, 0.4 ml/min.
[a] $n = 5$.
[b] $n = 2$.

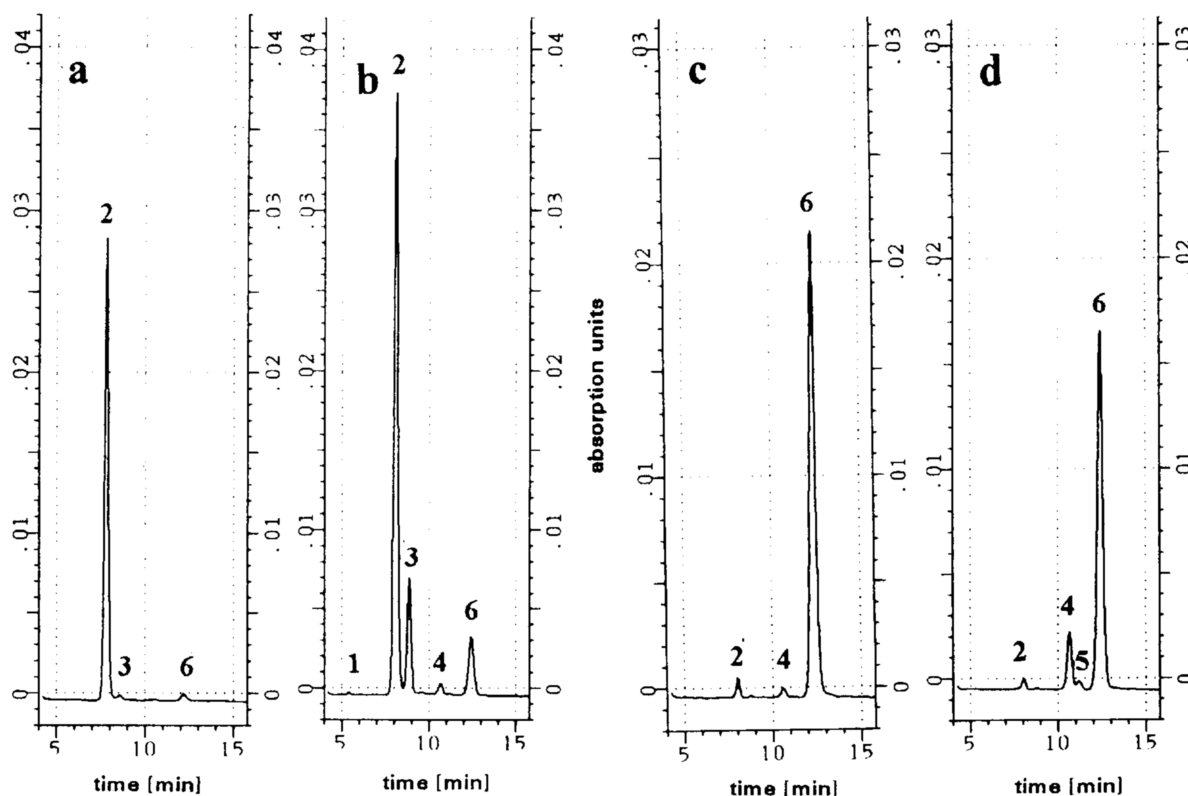

Fig. 1. Chromatograms obtained from untreated solutions of (a) 13-*cis*-retinol and (c) all-*trans*-retinol and (b, d) solutions of both isomers after photoisomerization. Stationary phase, 3-μm silica gel; column, 100 mm × 2 mm I.D.; mobile phase, 0.3% 1-octanol in *n*-hexane; flow-rate, 0.4 ml/min; detection wavelength, 325 nm. Peaks: 1 = 11-*cis*-; 2 = 13-*cis*-; 3 = 9,13-di-*cis*-; 4 = 9-*cis*-; 5 = 7-*cis*-; 6 = all-*trans*-retinol.

and all-*trans*-retinol are shown in Fig. 2. Table 2 shows the maximum absorption wavelengths obtained compared with literature data.

The HPLC system applied in this work allowed the separation of seven retinol isomers: 11-*cis*-, 11,13-di-*cis*-, 13-*cis*-, 9,13-di-*cis*-, 9-*cis*, 7-*cis*- and all-*trans*-retinol (see Fig. 3). Stancher and Zonta [15] achieved the resolution of even more isomers. They worked with a standard-sized HPLC column and a mobile phase of 3.9% 1-octanol in *n*-hexane. The separation of the 11-*cis*- and 11,13-di-*cis*-isomers was reversed in time compared with our results. The first-eluting peak was confirmed as the signal for 11-*cis*-retinol. According to Brown and Wald [16], on treatment of all-*trans*-retinyl palmitate with iodine, the 11-*cis*-isomer was formed as the main product. In our investigations, 77% of 11-*cis*-retinol relative to total retinol was found.

The system was calibrated with external standards ranging from 0.8 to 6.0 μg/ml of total retinol. The concentration of all-*trans*-retinol was determined by measuring a calibration graph at 325 nm, which showed linearity over three orders of magnitude of concentration. The correlation coefficient was 0.9998, the standard deviation of *y*-residuals (s_y) was 47 and the standard deviation of the *x*-value estimated using the regression line (s_{x0}) was 0.033 (regression line: $y = 1420x - 24$). The detection limit was 0.14 μg/ml and the determination limit was 0.42 μg/ml in the solution for analysis (or 1.4 μg per 100 g and 4.2 mg per 100 g of food sample, respectively). The repeatability of the method was

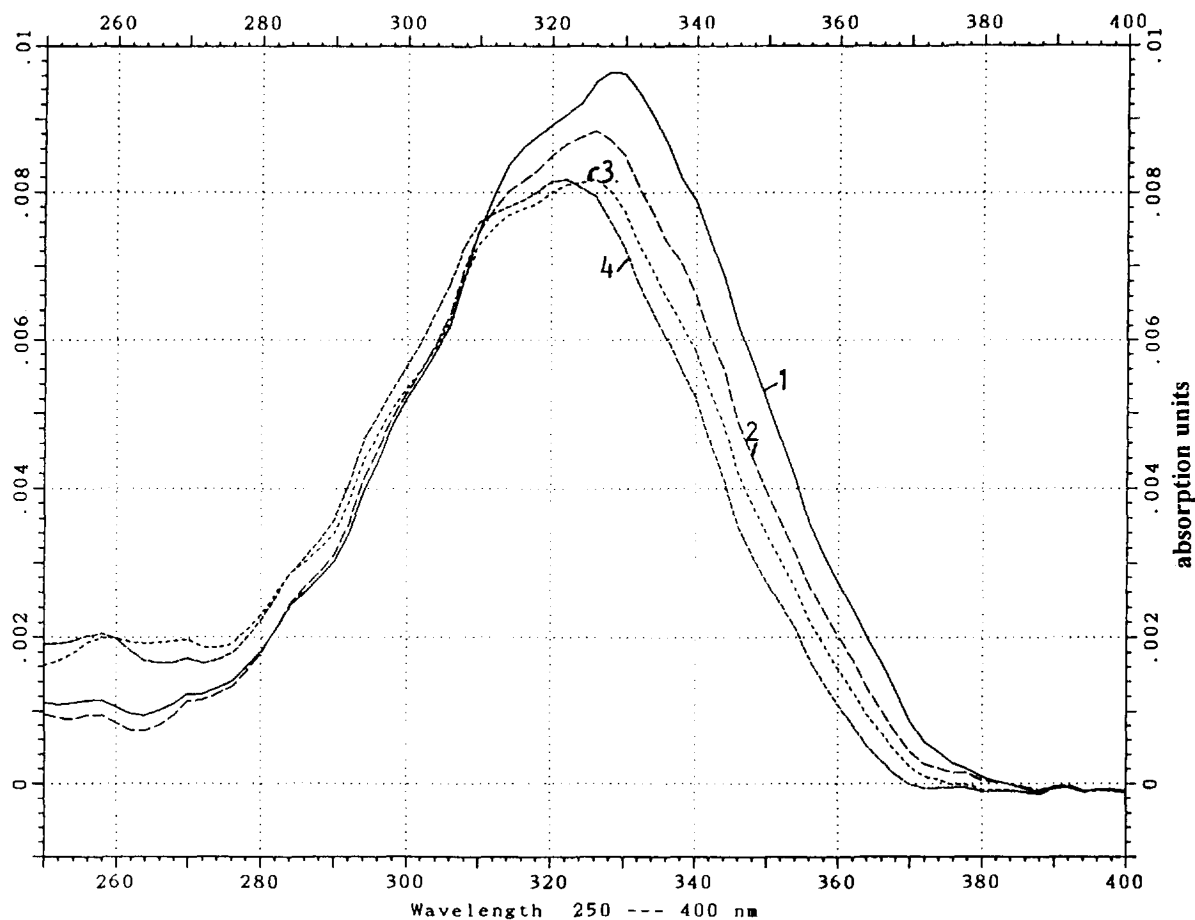

Fig. 2. Spectra of retinol isomers: 1 = 13-*cis*-retinol; 2 = all-*trans*-retinol; 3 = 9,13-di-*cis*-retinol; 4 = 9-*cis*-retinol.

Table 2
Absorption maxima of retinol isomers measured in *n*-hexane containing 0.3% 1-octanol compared with literature data [7,12]

Isomer	Maximum absorption wavelength (nm)		
	This work	Ref. [13]	Ref. [7]
11-*cis*-	322	322	319
11,13-Di-*cis*-	314	312	311
13-*cis*-	328	328	328
9,13-Di-*cis*-	324	324	324
9-*cis*-	322	323	323
7-*cis*-	314	322	No data available
All-*trans*-	325	326	325

tested by analysing different foods. For example, for beef liver the standard deviation was 0.3 mg per 100 g (2.2%) and for baby food 0.06 mg per 100 g (2.6%). Statistical data were calculated according to Funk et al. [17].

After confirming the signals for the seven isomers mentioned in Tables 1 and 2, the HPLC method was applied to extracts of the unsaponifiable matter of different food samples, such as livers of fattening animals in the raw state and after heating, liver-containing infant food in preservation vessels, liver sausages, different sorts of milk and one sample of sour cream.

As pure standards of most *cis*-isomers were not available, the percentage present in the

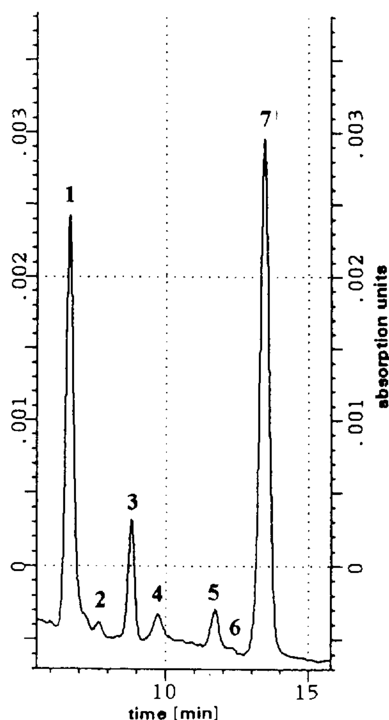

Fig. 3. Chromatogram obtained from an extract of an all-*trans*-retinyl palmitate standard solution after photoisomerization and saponification. Stationary phase, 3-μm silica gel; column, 100 mm × 2 mm I.D.; mobile phase, 0.3% 1-octanol in *n*-hexane; flow-rate, 0.4 ml/min; detection wavelength, 325 nm. Peaks: 1 = 11-*cis*-; 2 = 11,13-Di-*cis*-; 3 = 13-*cis*-; 4 = 9,13-*di-cis*-; 5 = 9-*cis*-; 6 = 7-*cis*-; 7 = all-*trans*-retinol.

samples had to be calculated. Two correction constants were applied for calculation: k_1 represents the ratio of the specific extinctions (ε) of the *cis*-isomers and all-*trans*-retinol and k_2 represents the ratio of the peak area measured at the maximum absorption wavelength to that at 325 nm. The specific extinction ratio has to be calculated as this value is lower for all *cis*-isomers than for all-*trans*-retinol. Measurement at only one detection wavelength (in our work 325 nm as the maximum absorption wavelength for the main isomer, all-*trans*-retinol, was applied) simplifies the method. To correct for the lower absorption of the *cis*-isomers at this wavelength, which differs from their maximum absorption wavelength (see Table 2), the peak-area ratio was calculated by recording three-dimensional chromatograms (absorption vs. time and wavelength) for various photoisomerized standard solutions. The evaluation system applied calculated the peak areas at every desired wavelength.

Table 3 shows k_1 and k_2. The constant k_1 was calculated making use of literature data on the specific extinctions [8]. Relative standard deviations are given for the calculation of k_2 (if sufficient data for calculating these values were available).

For calculation of the percentage of isomers, an equation using k_1 and k_2 was developed:

$$\text{Isomer}\ (\%) = \frac{A_{\text{isomer, 325 nm}} \cdot k_1 \cdot k_2}{\sum A_{\text{corrected}}}$$

where $A_{\text{isomer, 325 nm}}$ = peak area measured for the isomer peak at 325 nm, k_1 = correction constant representing the ratio of the specific extinctions (ε) of the *cis*-isomers and all-*trans*-retinol, k_2 = correction constant representing the ratio of the peak area measured at the maximum absorption wavelength of the *cis*-isomers to that at 325 nm and $\Sigma A_{\text{corrected}}$ = sum of peak areas that were corrected making use of k_1 and k_2 (denominator of the fraction) for all isomers occurring in the investigated sample.

The concentration of every isomer can be calculated from a knowledge of the all-*trans*-retinol concentration, which was determined with the external standard method.

Table 3

k_1 (ratio of specific extinctions of all-*trans*-retinol and *cis*-isomers calculated from literature data [7]) and k_2 [ratio of peak areas measured for the isomer signal at its maximum absorbance wavelength to that at 325 nm (measuring wavelength)]

Isomer	k_1	k_2	R.S.D. (%)
11-*cis*-	1.548	1.054	1.0
11,13-Di-*cis*-	2.031	1.119	–
13-*cis*-	1.093	1.013	0.6
9,13-Di-*cis*-	1.337	–	–
9-*cis*-	1.248	1.025	0.4
7-*cis*-	–	–	–
All-*trans*-	1.000	1.000	–

Conditions as in Table 1.

Table 4
Isomer distributions in liver and liver-containing products (%) and influence of thermal treatment

Food sample	11-*cis*-	11,13-Di-*cis*-	13-*cis*-	9,13-Di-*cis*-	9-*cis*-	7-*cis*-	All-*trans*-
Beef liver (raw)	n.d.[a]	n.d.	15.3	1.8	5.5	n.d.	77.4
Beef liver (after conventional heating)	n.d.	n.d.	16.1	3.3	8.4	n.d.	72.2
Beef liver (after microwave heating)	n.d.	n.d.	15.7	3.4	8.4	n.d.	72.5
Sheep liver (raw)	tr.[a]	tr.	9.3	1.6	5.5	n.d.	83.6
Pig liver (raw)	tr.	2.1	4.6	0.5	2.7	n.d.	90.1
Infant food (manufacturer 1)	4.7	n.d.	23.3	6.0	9.0	tr.	57.0
Infant food (manufacturer 2)	13.2	n.d.	3.9	tr.	2.7	tr.	80.2
Calf liver sausage	1.2	n.d.	8.7	1.3	3.2	tr.	85.6

[a] n.d. = Not detectable; tr = trace.

Tables 4 and 5 report on the isomer distributions of the types of food mentioned above. In all investigated types of liver-containing food, all-*trans*-retinol predominates (see Table 4). 13-*cis*-Retinol also occurred in every sample in detectable amounts. In two infant food samples from different manufacturers the 13-*cis*- and the 11-*cis*-isomers were present in considerable amounts. In one of them 13-*cis*-retinol amounted to 23.3% relative to total retinol. Possibly 11-*cis*-retinol in food may be formed by enzymes that are part of other ingredients. After treatment of a standard solution of all-*trans*-retinol palmitate with steapsine (pancreatic lipase), 6.2% of 11-*cis*-retinol relative to total retinol was found in the hydrolysed and extracted solution.

In raw liver samples of three species of animals, the retinol isomer pattern differed between the species. Only in raw pig liver did 11,13-di-*cis*-retinol occur in detectable amounts. The sample had been frozen for approximately 6 months at −18°C and had been warmed to room temperature before saponification and clean-up. In sheep liver only a trace of 11,13-di-*cis*-retinol could be demonstrated (see Table 4). Thermal treatment of beef liver with both microwave and conventional heating led to an increase in the percentages of *cis*-isomers, especially of 9,13-di-*cis*- and 9-*cis*-retinol.

In different types of milk that were available on the market, different isomer patterns were found (see Table 5). Untreated pasteurized milk contained 95.1% all-*trans*-retinol relative to total retinol whereas ultra-high temperature (UHT) milk contained more than 10% of *cis*-isomers. The preservation method in pasteurization is carried out at much lower temperature than in UHT treatment. After thermal treatment both with microwave and conventional heating the percentage of *cis*-isomers increased considerably in both kinds of milk. Fig. 4 shows chromatograms of UHT milk samples before and after

Table 5
Retinol isomer distribution in milk and dairy products (%) and influence of thermal treatment

Food sample	11-*cis*-	11,13-Di-*cis*-	13-*cis*-	9,13-Di-*cis*-	9-*cis*-	7-*cis*-	All-*trans*-
Pasteurized milk (untreated)	n.d.[a]	n.d.	4.9	n.d.	n.d.	n.d.	95.1
Pasteurized milk (after conventional heating)	tr.[a]	tr.	9.8	n.d.	5.4	n.d.	84.4
Pasteurized milk (after microwave heating)	tr.	tr.	6.8	n.d.	6.4	n.d.	86.6
UHT milk (untreated)	tr.	tr.	6.8	n.d.	3.6	n.d.	89.6
UHT milk (after conventional heating)	tr.	tr.	12.3	2.5	5.3	n.d.	79.9
UHT milk (after microwave heating)	tr.	tr.	14.6	3.2	7.5	n.d.	74.7
Pasteurized milk (fermented)	9.8	tr.	9.0	0.8	3.1	tr.	77.3
Sour cream	7.3	tr.	3.7	tr.	2.3	n.d.	86.7

[a] n.d. = Not detectable; tr = trace.

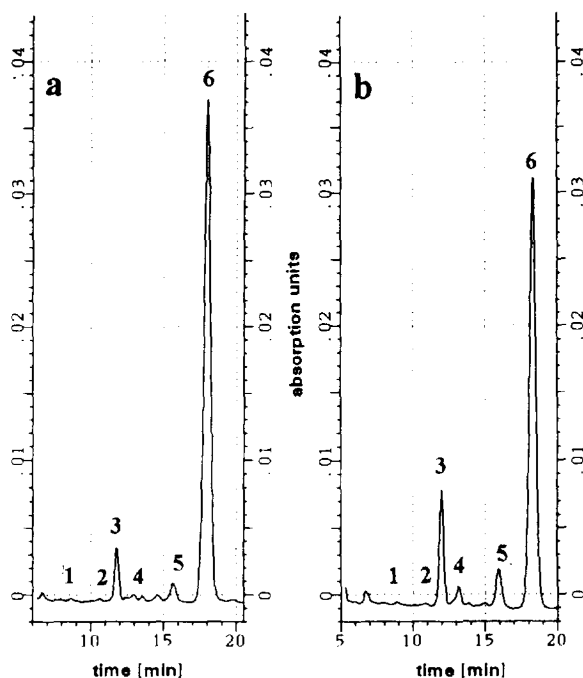

Fig. 4. Chromatograms obtained from extracts of the unsaponifiable matter of (a) untreated and (b) microwave-heated UHT milk. Stationary phase, 3-μm silica gel; column, 100 mm × 2 mm I.D.; mobile phase, 0.25% 1-octanol in *n*-hexane; flow-rate, 0.4 ml/min; detection wavelength, 325 nm. Peaks: 1 = 11-*cis*-; 2 = 11,13-di-*cis*-; 3 = 13-*cis*-; 4 = 9,13-di-*cis*-; 5 = 9-*cis*-; 6 = all-*trans*-retinol.

thermal treatment. In unfermented dairy products only traces of 11,13-di-*cis*- and 11-*cis*-retinol were detectable (see Table 5). After leaving a sample of pasteurized milk at room temperature for several days so that it became sour as a result of microbiological fermentation, a considerably high percentage of 11-*cis*-retinol (9.8% relative to total retinol) was found. In sour cream 7.3% 11-*cis*-retinol relative to total retinol was found.

As all *cis*-isomers possess different vitamin A activities lower than that of all-*trans*-retinol, it is important to know the isomer distribution in food samples. Values of the vitamin A activities of the isomers can be obtained from the literature [8]. 13-*cis*-Retinol possesses 75%, 9,13-di-*cis*-retinol 24%, 9-*cis*-retinol 22%, 11-*cis*-retinol 23% and 11,13-di-*cis*-retinol 15% of the activity of all-*trans*-retinol.

Heating and processing foods result in losses of vitamin A activity by two mechanisms: degradation of retinol and isomerization of all-*trans*-retinol into *cis*-isomers. For liver and milk, investigations were made concerning the losses of total vitamin A and the vitamin A activity calculated by making use of the activity values obtained from literature. Isomer distributions are given in Tables 4 and 5. Vitamin A losses during different heating processes are given in Table 6. Heating of milk led to vitamin A losses between 6.8 and 9.8% whereas the activity losses ranged between 9.5 and 16.0%, as measured by the increase in *cis*-isomers. Also, heating of liver results in vitamin A activity losses higher than the loss of total vitamin A (see Table 6).

4. Conclusions

Applying normal-phase HPLC using narrow-bore columns of 100 mm × 2 mm I.D., good

Table 6
Losses of total vitamin A and vitamin A activity losses in milk and liver during different heating processes

Food sample[a]	Loss of total vitamin A (%)	Vitamin A activity loss (%)
Pasteurized milk (MW)	6.8	9.5
Pasteurized milk (conv.)	9.3	14.2
UHT milk (MW)	9.8	16.0
UHT milk (conv.)	7.1	11.1
Beef liver (MW)	19.1	22.6
Beef liver (conv.)	32.7	35.6

[a] MW = microwave heating, conv. = conventional heating (mass losses after heating processes were considered).

separation results for retinol isomers were achieved. Low percentages of 1-octanol in *n*-hexane as mobile phase allowed the separation of *cis*-retinols and all-*trans*-retinol in photoisomerized standard solutions and also in extracts of the unsaponifiable matter of diverse food samples.

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