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Improved simultaneous determination method of β-carotene and retinol with saponification in human serum and rat liver

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Abstract

Among the many simultaneous determination methods for carotenoid and retinoid, there are only a few reports including the saponification process. However, the yields of β-carotene and retinol were higher when using this process. In this study, the analytical conditions, including saponification, were investigated. The extraction solvent was *n*-hexane and the sample solvent was HPLC mobile phase in the β-carotene and retinol analysis. BHT as an antioxidant was added at concentrations of 0.125 and 0.025%, respectively, to ethanol and *n*-hexane phase in the extraction process for serum. The recovery rates were 99.7, 93.7 and 98.3% for β-carotene, retinol and retinyl palmitate in serum, respectively, and 107.1, 92.8 and 98.8% for β-carotene, retinol and retinyl palmitate in liver, respectively. The within-day coefficients of variation (C.V.) were 6.0% for serum and 4.7% for liver in the case of β-carotene, 7.1% for serum, and 5.1% for liver in the case of retinol. The between-day coefficients of variation were 2.7% for serum and 2.7% for liver in the case of β-carotene, and for retinol, 6.4% for serum and 2.7% for liver.

Keywords: Saponification; β-Carotene; Retinol

1. Introduction

Recently, carotenoids possessing antioxidative properties and immunity functions have attracted the attention of the general public and researchers in the food and nutrition field. Similarly, retinoids with anticarcinogenic and morphologic actions are of major interest [1–4]. The relationships between the amounts of these substances and their functions in tissues have been reviewed by Menkes et al. [5]. It is well known that several carotenoids such as β-carotene are inverted to retinal in the intestinal mucosa of animals and these carotenoids are of the pro-vitamin A type (Fig. 1). For research purposes, it is important to determine, as accurately and simultaneously as possible, the amounts of carotenoids and retinoids absorbed and metabolized in bodily organs.

Many procedures have already been reported for the simultaneous determination of carotenoids and retinoids [6–10]. However, the saponification process was not carried out in the simultaneous de-

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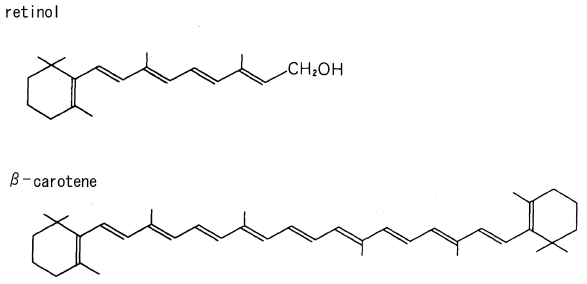

Fig. 1. Structures of β-carotene and retinol.

termination methods in many previous papers and only a few determination methods include saponification [11–14]. In this experiment, the measured amounts of β-carotene in the liver and serum using the saponification process were larger than without this process, and the amount of retinol in serum using the saponification process was greater than without this process. We report an improved simultaneous determination method for β-carotene and retinol in the liver and serum using the saponification process, and thoroughly examine the analytical conditions of this method.

2. Experimental

2.1. Chemicals

All-*trans* β-carotene, retinol and retinyl palmitate samples were purchased as standard substances from Sigma (St Louis, MO, USA). Ethanol, methanol, *n*-hexane, ethyl acetate, chloroform, acetonitrile, dichloromethane, toluene and isopropanol were purchased (special grade) from Katayama (Osaka, Japan). *tert*.-Butyl hydroxy toluene (BHT) was purchased (special grade) from Wako (Osaka, Japan).

2.2. Preparation of serum and liver samples

Human blood was centrifuged for 20 min at 1900 *g* and its supernatant was stored as serum at −40 °C. Rat livers were rinsed in a saline solution, wiped with filter paper and then stored at −40 °C until required for assay. One gram of liver was homogenized with 10 ml of water using a Waring blender (10 000 rpm, 5 min) and its homogenates were used in the determination procedure.

2.3. Extraction and HPLC analysis

β-Carotene and retinol were extracted by modifying a method reported by Shapiro et al. [11]. In this, 0.15 ml of serum was mixed with water (0.35 ml), 3% sodium ascorbate solution (0.5 ml) and ethanol (2.0 ml, containing 0.125% BHT). After incubation at 70 °C for 5 min, samples were saponified with 10 *M* KOH (1.0 ml) and heated at 70 °C for 30 min. After cooling, hexane (4.0 ml) (containing 0.025% BHT) was added. The tubes were then vigorously machine shaken for a 10-min period and centrifuged (500 *g*, 10 min). This extraction process was repeated three times. The *n*-hexane extracts were dried by N_2 gas at 40 °C and the residue was dissolved using 300 μl of the HPLC mobile phase for HPLC analysis. Liver analysis proceeded by mixing 25% sodium ascorbate (0.5 ml) with homogenate (0.3 ml) and water (0.7 ml). Ethanol (2.0 ml) was then added. The liver sample was extracted under exactly the same saponification and extracting procedure. However, for the liver sample, BHT was not added. The HPLC analysis was carried out using a PU-890 Intelligent Pump and an automated injection system (851-AS, Japan Spectroscopic, Tokyo, Japan). The column and HPLC mobile phase were used according to Barua's conditions [15]. The column employed for HPLC determination was a Resolve 5-μm spherical C_{18} column (150×3.9 mm, Waters, Milford, MA, USA). The mobile phase was acetonitrile–dichloromethane–methanol–1-octanol (90:15:10:0.1 v/v) at 1.0 ml/min. The column oven (TU-100, Japan Spectroscopic) was set at 30 °C. The detector used for the determination of β-carotene was a visible detector (870UV, Japan Spectroscopic) set at 451 nm. An ultraviolet detector (UVIDEC-III, Japan Spectroscopic) was set at 325 nm to measure the retinol. Signals from both detectors were recorded simultaneously by the data system (SIC-480, System Instrument, Tokyo, Japan). The fundamental process

of the simultaneous determination of β-carotene and retinol is shown in Fig. 2.

2.4. Examination of saponification, extraction solvent, sample solvent and antioxidant

The effect of saponification and the antioxidant was investigated according to the modified extraction and HPLC analysis procedures described above. Furthermore, the measured amounts of β-carotene and retinol obtained using various solvents which extract β-carotene and retinol from serum and liver homogenate were compared. These measured amounts were compared using various sample solvents which dissolve the extract residues. *n*-Hexane, chloroform and ethyl acetate were used as the extraction solvents. Methanol, ethanol, methanol–dichloromethane (6:4), methanol–toluene (5:5), *n*-hexane, ethyl acetate, chloroform, isopropanol and HPLC mobile phase were used as the sample solvents.

2.5. Accuracy

The accuracy was evaluated by standard addition recoveries. The known amounts of β-carotene, retinol or retinyl palmitate dissolved in *n*-hexane, methanol, or methanol, respectively, were added to the serum and the liver homogenate, before saponification. These substances were then extracted using the above described method.

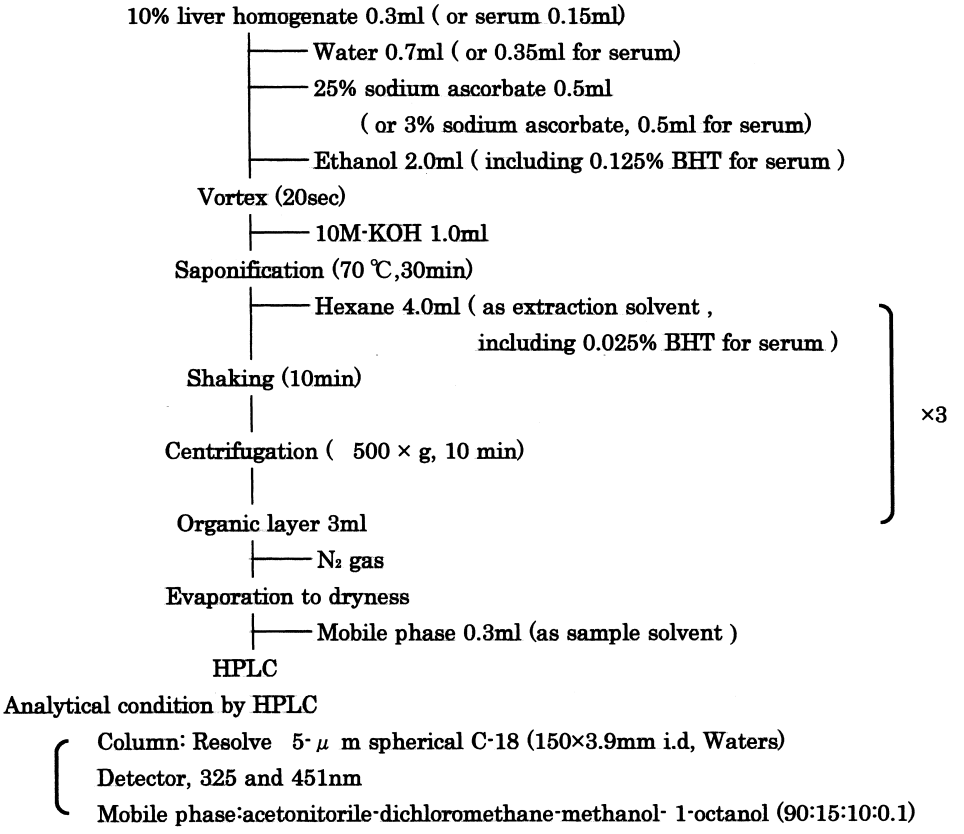

Fig. 2. Improved simultaneous determination method for β-carotene and retinol.

2.6. Precision

To assess the precision of the method, the serum and liver samples were analyzed six times on the same day (within-day precision), and the same samples were extracted and analyzed six times for three consecutive days (between-day precision).

2.7. Calibration solution and calculation

Individual stock solutions were prepared using pure standard β-carotene and retinol stored at −20 °C. β-Carotene was dissolved in *n*-hexane. Retinol was dissolved in methanol. The calibration of the β-carotene and retinol concentration was based on a working curve method.

2.8. Statistical analysis

The results were subjected to Student's *t*-test and one-way ANOVA. Differences in the mean values among the groups were tested using Fisher's least significant difference procedures.

3. Results and discussion

3.1. HPLC chromatograms of β-carotene and retinol

Chromatograms for β-carotene and retinol in standard solutions, prepared human serum and rat liver solutions are shown in Fig. 3.

3.2. Standard curves

Known amounts of standard were added to either human serum or rat liver homogenate to investigate the matrix effect. The calibration curve slopes and coefficients of the standard solution and standard addition solution are presented in Table 1. Linearity was established by least-squares regression analysis. The calibration curve slopes were identical between the direct and standard addition solutions. The correlation coefficients showed a similarity of the linear relationship between the amount of each compound and their peak heights ($r>0.999$). The linear concentration ranged from 0.16 to 16.09 μmol/l for β-carotene and from 0.09 to 46.03 μmol/l for retinol. The lowest detection limits were 0.124 μmol/l for β-carotene, and 0.089 μmol/l for retinol with an injection volume of 20 μl.

3.3. Effect of saponification

It has been reported [16] that the measured values of carotenoids by saponification were smaller than those without saponification. However, in other papers [13,14,17,18], the determination of carotenoid requires saponification. In the simultaneous determination of β-carotene and retinol, the saponification step has not been included in the majority of previous papers [15,16,19–22] with a few exceptions [12]. When saponification was carried out according to Shapiro's method [11], the resulting amount of β-carotene in the serum and liver was significantly greater than that compared with non-saponification methods (Table 2). As shown in Table 2, β-carotene in the liver increased 1.5 times in the saponification method, while the amount of β-carotene in the serum increased by a factor of 2.7. Furthermore, the measured values of serum retinol by saponification were 1.2 times greater than without saponification. It was thought that the saponified β-carotene and retinol in the liver and serum were extracted efficiently by the extraction solvents. These results show that the saponification procedure was necessary to determine β-carotene and retinol.

3.4. Extraction solvent

n-Hexane [11–14,16,19–27], chloroform [28], and ethyl acetate [15] were all used as extraction solvents in this experiment in order to select the most suitable extraction solvent (Table 3). The measured values of β-carotene in the serum and liver using *n*-hexane were significantly higher than with ethyl acetate or chloroform. The amount of serum retinol extracted by *n*-hexane was significantly greater than with chloroform, and greater still when extracted using ethyl acetate. The higher amount of liver retinol extracted by *n*-hexane was significantly more than with ethyl acetate and greater than the amount extracted with chloroform. This experiment added support to other reports that revealed *n*-hexane to be the most suitable extraction solvent.

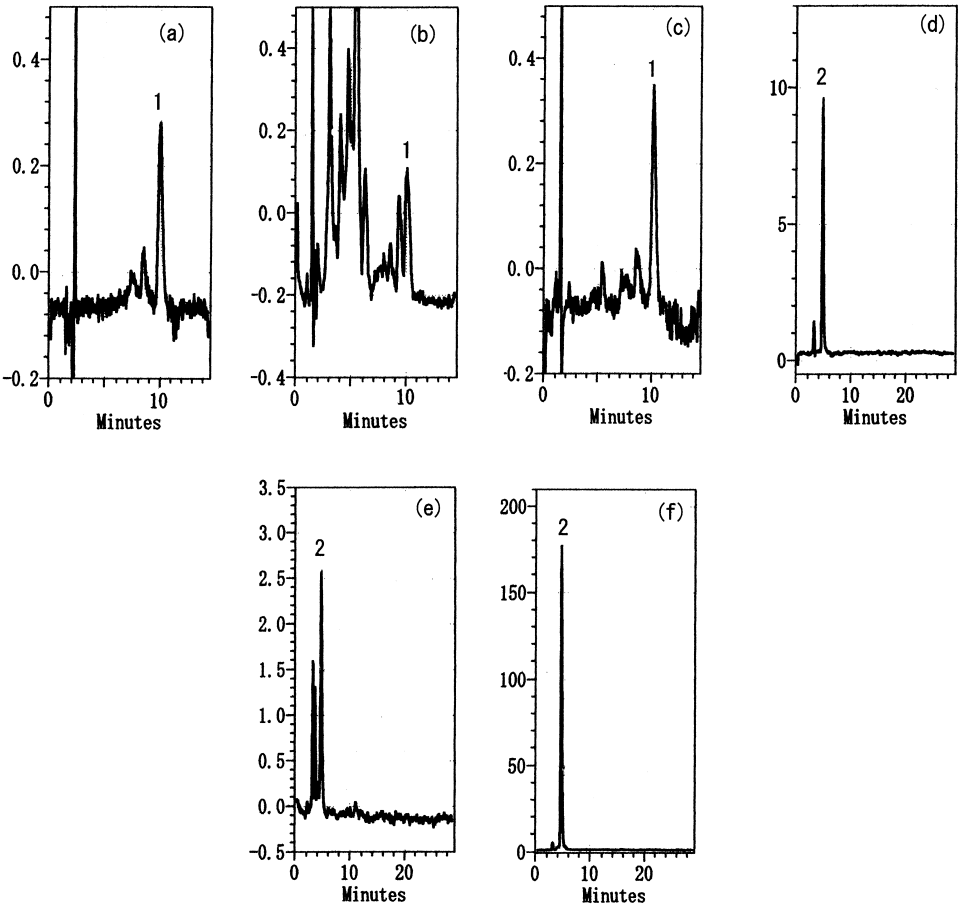

Fig. 3. Chromatograms of β-carotene and retinol. (a) Standard β-carotene (0.35 μg/ml); (b) human serum; (c) rat liver; (d) standard retinol (0.26 μg/ml); (e) human serum; (f) rat liver. Peaks: 1=β-carotene; 2=retinol. HPLC column: Resolve 5 μm spherical C_{18} column (150 mm×3.9 mm I.D.), mobile phase: acetonitrile–dichloromethane–methanol–1-octanol (90:15:10:0.1), flow-rate: 1.0 ml/min, detection: (for β-carotene) 451 nm; (for retinol) 325 nm.

Table 1
Linearity of β-carotene and retinol

Compound			LR	*r*
β-Carotene	Human serum	Standard addition	$y=0.0057x+0.1075$	0.9999
		Direct	$y=0.0055x+0.0087$	0.9996
	Liver	Standard addition	$y=0.0058x+0.0599$	0.9991
		Direct	$y=0.0055x+0.0087$	0.9996
Retinol	Human serum	Standard addition	$y=0.0229x+3.7378$	0.9966
		Direct	$y=0.0228x+0.0507$	1.0000
	Liver	Standard addition	$y=63.20x+95.09$	0.9990
		Direct	$y=63.58x+2.87$	1.0000

LR, Linear regression; r=coefficient of correlation; y=peak-height; x=β-carotene or retinol concentration (ng or μg). Direct: standard solution only; standard addition: standard solution+human serum (0.15 ml) or 10% rat liver homogenate (1.0 ml). Linear concentration ranges (μmol/l) were 0.16–16.09 for β-carotene and 0.09–46.03 for retinol, respectively.

Table 2
Effect of saponification treatment on the amounts of β-carotene and retinol in human serum and rat liver

Treatment	β-Carotene		Retinol
	Serum (μg/l)	Liver (μg/g)	Serum (μg/l)
Saponification	586.9±9.8	0.53±0.05	543.4±24.0
Non-saponification	220.8±42.3**	0.36±0.05**	458.0±27.1**

Values are means±SD (n=4). **P<0.01.

Table 3
Comparison of the amounts of β-carotene and retinol by various extraction solvents

	Serum (μg/l)		Liver (μg/g)	
	β-Carotene	Retinol	β-Carotene	Retinol
n-Hexane	458.3±21.2[a]	294.5±13.3[a]	0.53±0.04[a]	42.87±1.43[a]
Ethyl acetate	375.2±28.4[b]	273.8±28.5[a]	0.33±0.05[b]	31.02±2.31[b]
Chloroform	258.6±48.8[c]	218.6±32.2[b]	0.39±0.05[b]	39.98±2.67[a]

Values are means±SD (n=4). Values with different superscript letters are significantly different (P<0.05).

3.5. Sample solvent

The following sample solvents were used in this experiment: methanol [18,24,28], ethanol [12,16,27], methanol–dichloromethane (6:4) [15], methanol–toluene (5:5) [11], *n*-hexane [13], ethyl acetate, chloroform, isopropanol [23] and mobile phase [13,14,19,22,25,26] (Table 4). The amounts of β-carotene and retinol extracted from the serum using mobile phase, methanol, ethanol, methanol–dichloromethane (6:4) and methanol–toluene (5:5) were greater than those with other sample solvents. The determined amount of β-carotene and retinol extracted from the liver was higher when using HPLC mobile phase and methanol–toluene (5:5). The mobile phase in HPLC analysis has been used as a sample solvent in many reports [13,14,19,22,25,26], while methanol [18,24,28] and ethanol [12,16,27] have been used in fewer studies. Epler et al. [21] reported that the amounts of carotenoids in human serum using ethanol and ethyl acetate as sample solvents were greater. Furthermore, the mobile phase solvent was suitable for the simultaneous assay of β-carotene and retinol in this experiment.

Table 4
Comparison of the amounts of β-carotene and retinol by various sample solvents

Sample solvent	Serum (μg/l)		Liver (μg/g)	
	β-Carotene	Retinol	β-Carotene	Retinol
Mobile phase	887.7±46.1[a]	457.4±19.1[a]	0.67±0.02[a]	57.85±1.50[a]
Methanol	823.6±25.4[a]	462.3±60.3[a]	0.56±0.07[b]	55.31±0.93[a,b]
Ethanol	838.3±22.3[a]	444.0±11.9[a]	0.58±0.02[b,c]	53.62±3.55[b,c]
Methanol–dichloromethane (6:4)	879.5±17.6[a]	492.1±26.7[a]	0.67±0.03[a]	48.67±1.60[d]
Methanol–toluene (5:5)	788.3±13.6[a]	445.0±98.0[a]	0.60±0.03[b,c,d]	55.43±1.64[a,c]
n-Hexane	677.5±35.3[b]	432.5±45.1[a]	0.56±0.02[b,c,d]	48.31±2.09[d]
Ethyl acetate	805.3±130.6[a]	278.5±15.9[b]	0.53±0.02[b]	47.10±0.93[d]
Chloroform	559.3±52.7[b]	195.9±39.7[c]	0.31±0.03[e]	45.89±0.56[d]
Isopropanol	878.5±59.8[a]	310.3±25.4[b]	0.55±0.04[b,c]	56.03±4.85[a]

Values are means±SD (n=4). Values with different superscript letters are significantly different (P<0.05).

3.6. Effect of antioxidants

In previous papers, the BHT was added to the ethanol phase [15,16] of the extraction process or extraction solvent [22], or to both the ethanol phase and extraction solvent [20,21,28], and the antioxidant effect of BHT was either recognized [29] or not [19]. The effect of BHT concentration as taken from Chow and Omaye [29] on the β-carotene and retinol amounts measured in the serum and liver was investigated (Table 5). BHT was added to the ethanol phase (0, 0.125 and 0.250% concentrations) and to the *n*-hexane phase (0, 0.025 and 0.050% concentration). The BHT levels of both the ethanol phase and the *n*-hexane phase barely affected the amount of β-carotene in the serum and liver. The measured amount of retinol in the serum was significantly increased by the addition of BHT into the ethanol phase (0.250%) and *n*-hexane phase (0.050%), but it decreased in the liver at both low and high BHT levels in the ethanol phase and *n*-hexane phase.

The results of this experiment have shown that the addition of BHT into the ethanol phase and extraction solvent [29] was necessary for the serum retinol assay, but were not necessary for the β-carotene and retinol assay from the liver (because of a decrease in these measured values). It was thought that the cause of this decrease was the addition of a large amount of ascorbic acid into the liver homogenate. The recovery of both retinol and tocopherol in rat serum by simultaneous determination increased with the addition of BHT to the ethanol phase and extraction solvent [29]. However, tocopherol recovery was reduced by the addition of ascorbic acid to the ethanol phase and similarly, BHT to the extraction solvent. Therefore, BHT was added to the ethanol phase at 0.125% and to the extraction solvent at 0.025% in the serum assay, but BHT was not added in the liver assays.

Table 6
Recovery of β-carotene and retinol in human serum and rat liver

		Recovery (mean±SD) (%)
Serum		
β-Carotene		99.7±3.0
Retinol	Added retinol	93.7±6.6
	Added retinyl palmitate	98.3±5.3
Liver		
β-Carotene		107.1±6.2
Retinol	Added retinol	92.8±7.8
	Added retinyl palmitate	98.8±7.3

Values are means±SD (n=4).

3.7. Recovery of β-carotene and retinol

In the case of β-carotene, the recovery process yielded 99.7±3.0% from the serum and 107.1±6.2% from the liver (Table 6). These yields of retinol and retinyl palmitate from the serum were 93.7±6.6% and 98.3±5.3% expressed as retinol values, respectively. The recovery of retinol and retinyl palmitate from the liver was 92.8±7.8% and 98.8±7.3% as retinol values, respectively.

3.8. Precision

As shown in Table 7, in the case of β-carotene, the within-day coefficients of variation (C.V.) were

Table 5
Effect of an antioxidant on the amounts of β-carotene and retinol in human serum and rat

BHT concentration		β-Carotene		Retinol	
Ethanol (%)	*n*-Hexane (%)	Serum (μg/l)	Liver (μg/g)	Serum (μg/l)	Liver (μg/g)
0	0	482.6±15.9	0.39±0.01	522.1±51.2[a]	48.49±2.34[a]
0.125	0.025	470.6±38.2	0.38±0.03	586.8±22.4[a,c]	44.70±3.25[b]
0.250	0.050	457.0±12.8	0.37±0.01	609.1±87.5[b,c]	44.29±1.71[b]

Values are means±SD (n=4). Values with different superscript letters are significantly different (P<0.05).

Table 7
Variability of β-carotene and retinol in human serum and rat liver

Analyte	Within day		Between days	
	Mean±SD[a]	CV (%)	Mean±SD[b]	CV (%)
Serum				
β-Carotene	393.2±23.5	6.0	383.9±10.3	2.7
Retinol	606.7±43.3	7.1	600.8±38.3	6.4
Liver				
β-Carotene	0.85±0.04	4.7	0.75±0.02	2.7
Retinol	78.42±3.98	5.1	73.87±2.02	2.7

The means for serum are expressed in μg/l. The means for liver are expressed in μg/g.
[a] Mean of six samples.
[b] Mean of six duplicate assays.

6.0% for serum and 4.7% for liver, and in the case of retinol, 7.1% for serum and 5.1% for liver. The between-day coefficients of variation, in the case of β-carotene, were 2.7% for serum and 2.7% for liver, and for retinol, 6.4% for serum and 2.7% for liver.

4. Conclusion

This method can be considered a relatively simple, stable and accurate procedure for the routine simultaneous determination of β-carotene and retinol in serum and the liver, with the sample preparation and analytical time being relatively short. The measured amounts of β-carotene and retinol in the serum and liver according to the saponification method were higher than without the saponification method. In the simultaneous determination method for β-carotene and retinol, high measured amounts of these substances were obtained when the extraction solvent was *n*-hexane, the sample solvent was its mobile phase and BHT as an antioxidant was used only in serum analysis. The high measured amounts of these substances using the saponification method showed that the β-carotene and retinol in these samples were very soluble in this process. These results show that *n*-hexane as an extraction solvent has a higher extraction efficiency for β-carotene and retinol from serum and liver samples, and also, its mobile phase as a sample solvent has a high solubility for these substances in the extraction residue. The high measured amounts of these substances in serum with the addition of BHT as an antioxidant showed that their oxidation was inhibited.

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