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Short communication

Rapid high-performance liquid chromatographic method for determination of β-carotene in milk

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Abstract

An HPLC method for the determination of β-carotene in milk samples is described. The method has many advantages over previous methods due to its rapidity, convenience and the small volumes of solvents required. β-Carotene was 100% recovered and the relative standard deviation of repeatability was 5.3%. The limit of quantitation was found to be about 30 ng/ml and the system was linear in the concentration range investigated, from 0.67 to 4 μg/ml.

1. Introduction

β-Carotene is commonly known as a radical scavenger and a physical scavenger of singlet oxygen and is believed to play an important role in the inhibition of initial stages of lipid peroxidation. β-Carotene could thus be expected to play an important role in the oxidative stability of milk fat [1]. The β-carotene content of different foods is of nutritional importance and of additional interest since β-carotene is believed to have a protective role against cancer. β-Carotene is also of interest in relation to the reproduction performance of dairy cows [2].

Over the past decade, several methods have been used for determination of β-carotene in milk [3–6]. Methods applied until recently have used large to very large volumes of solvents [3–5]. However, one of the published methods does not use large volumes of solvents and no saponification is performed [6].

The aim of this investigation was to find a rapid and more convenient method using only small volumes of solvents. The method described in this work is suitable for determination of β-carotene in milk samples and lipid extracts of dairy products in general.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. The β-carotene standard, 97% (UV) was purchased from Fluka (Buchs, Switzerland). The purity of the β-carotene standard was checked by HPLC and was found to be 82%, assuming equal detector response factors for impurities as for β-carotene. α-Carotene was obtained from

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Fluka and lutein was a gift from Roche (Stockholm, Sweden).

2.2. HPLC conditions

The HPLC system consisted of a SP 8700 solvent delivery system and a SP 8700 pump (Spectra-Physics, San Jose, CA, USA), a Rheodyne 7125 injector fitted with a 10-μl loop, a Lambda-Max Model 480 UV detector (Waters Associates, Milford, MA, USA) set at 450 nm and a HP 3390 A integrator (Hewlett-Packard, Avondale, PA, USA). Two ChromSep ChromSpher PAH glass columns (10 cm × 3 mm I.D., particle size 5 μm, Chrompack, Netherlands) connected in series were used and the mobile phase was acetonitrile–methanol–dichloromethane (80:14:6, v/v) at a flow-rate of 0.7 ml/min. The β-carotene peak was identified and quantified against an external standard.

2.3. Sample preparation

Extraction

Milk samples were extracted essentially as for rehydrated milkpowder described by Nourooz-Zadeh and Appelqvist [7]; a portion of 6 ml isopropanol was added to 3 ml milk and the mixture was vortexed. Then the mixture was extracted twice with 4.5 ml hexane and centrifuged for phase separation. The pooled hexane extracts were washed once with 3 ml 0.47 *M* aqueous sodium sulphate and evaporated under nitrogen until ca. 2 ml of the hexane remained. The lipid content of the extract was determined by weighing on an ultra microbalance (UMT2, Mettler-Toledo, Greifensee, Switzerland).

Saponification

All extracted samples, except for one, were saponified prior to HPLC analysis using the following method. Portions of the hexane extracts (containing 50–100 mg lipids) were evaporated under nitrogen and dissolved in 1 ml ethanol (95%, v/v, containing 12.5 mg/ml pyrogallol as an antioxidant). Then, 1 ml of KOH (60% in water, w/v) was added. The samples were left for 30 min at 30°C with occasional vortexing. One ml of distilled water was added and the samples were extracted three times with 2 ml hexane and centrifuged to obtain phase separation. The hexane phases were pooled and taken to dryness under nitrogen. Prior to HPLC analysis, the samples were dissolved in 300 μl hexane–dichloromethane (95:5, v/v). The saponification was checked by TLC [silica gel plate; solvent system: hexane–diethyl ether (70:30, v/v); spray: 10% phosphomolybdenic acid in diethyl ether–ethanol (1:1, v/v)]. No traces of triglycerides were found after 30 min saponification at 30°C.

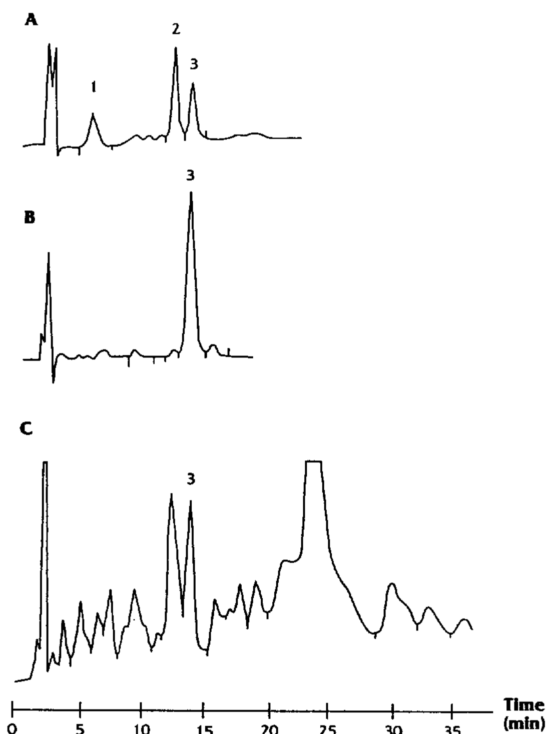

Fig. 1. (A) Separation of lutein (1), α- (2) and β-carotene (3) on two ChromSep ChromSpher PAH glass columns (10 cm × 3 mm I.D., particle size 5 μm, Chrompack, Netherlands) connected in series. The mobile phase was acetonitrile–methanol–dichloromethane (80:14:6, v/v) at a flow-rate of 0.7 ml/min. The detector was set at 450 nm. (B, C) Comparison between chromatograms of β-carotene (3) for a saponified sample (B) and a non-saponified sample (C).

3. Results and discussion

The chromatographic system was linear within the concentration range investigated, from 0.67 to 4 μg/ml, the higher concentration corresponding to a concentration in milk of about 24 μg/g milk fat. The limit of quantitation was found to be about 30 ng/ml. Repeatability of the complete method – extraction, saponification and HPLC analysis – was found to be 5.3% R.S.D., determined by extraction of three parallel milk samples, each saponified in duplicate and analysed with a single HPLC injection. Recovery was found to be 100% ± 6, determined in four parallel milk samples spiked with β-carotene to a final concentration of 3.6 μg/ml milk.

The HPLC separation of a carotene standard mix containing lutein, α- and β-carotene is shown in Fig. 1A. The reversed-phase system in this paper is basically very similar to those used earlier by Ollilainen et al. [4] and Indyk [5] and suitable for determination of β-carotene.

A compilation of the basic parameters of methods in the literature using saponification and the method described in this paper is presented in Table 1. Compared with methods published earlier the new method is easier to use. Sample preparation can be carried out in small tubes, since only small solvent volumes (5 ml) are needed for saponification and extraction. This is a considerable advantage over previous methods using volumes between 40 and 350 ml, and where a separatory funnel is necessary for the extraction after saponification. An additional feature is the use of hexane alone rather than a two-component solvent mixture.

In the new method, saponification is carried

Table 1
Characteristics of earlier methods using saponification, and the new method described in this paper

	Ollilainen et al. [4]	Indyk [5]	Panfili et al. [3]	New method
Sample	Milk (5–10 g)	Milk (5 ml) or powder	Cheese (0.5 g)	Milk fat (50–100 mg)
Saponification	40 ml water containing 2.5% vitamin C	10 ml ethanol containing 0.1% vitamin C	5 ml ethanol containing 6% pyrogallol	1 ml ethanol containing 1.25% pyrogallol
	10–40 g KOH–water (1:1, w/v)	2 ml KOH (50% in ethanol)	2 ml KOH (60% in ethanol)	1 ml KOH (60% in ethanol)
	100 ml ethanol		2 ml ethanol	
			1 ml NaCl (1%)	
Conditions	Room temperature 16 h	70°C 10 min	70°C 30 min	30°C 30 min
Extraction	100 ml NaCl (10%)	30 ml water	15 ml NaCl (1%)	1 ml water
	10 drops butylated hydroxytoluene, BHT (0.1%)			
	hexane-diethyl ether (70:30) (3 × 100 ml)	Light petroleum (b.p. 60–80°C)–diisopropyl ether (75:25) containing 0.01% BHT (20 ml)	hexane–ethyl acetate (9:1) (2 × 15 ml)	hexane (3 × 2 ml)
Recovery	100%	96–97%	99%	100%

out at 30°C for 30 min (100% recovery). Ollilainen et al. [4] also use mild conditions, but for as long as 16 h (100% recovery). Indyk [5] and Panfili et al. [3] both use saponification at 70°C for 10 min (96–97% recovery) and 30 min (99% recovery), respectively. However, the results of the new method demonstrate that there is no need to use a time/temperature combination more powerful than 30 min at 30°C for saponification of milk fat.

Thompson et al. [6] did not use saponification prior to the HPLC analysis of vitamin A and β-carotene in dairy products, mainly because of losses of vitamin A during saponification. Since, in the method described here, the recovery of β-carotene was found to be 100% and since the life time of the column is extended when injecting saponified samples compared to crude samples, a method including saponification is preferable. It was also found that the detector used in this study was sensitive to the so-called "second order effect". This will be present as a problem when the operating wavelength is higher than 350 nm and will give a less intense energy band at half the wavelength used. In the case of β-carotene analysis, this means that absorbance around 225 nm will disturb the analysis and this is where most of the lipids absorb. The "second order effect" made it necessary to saponify the samples prior to analysis, since a "forest" of interfering peaks were found in the β-carotene area in the chromatogram. A comparison between a saponified and a non-saponified sample analysed with our HPLC conditions is shown in Fig. 1B and C.

In conclusion, a modified method for the determination of β-carotene in milk has been described. The method uses small volumes of solvent and mild saponification conditions and is therefore rapid, economical and convenient to use. Most probably the method is also applicable to lipid extracts of other dairy products.

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