Variation in retinol and carotenoid content of milk and milk products in The Netherlands

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Abstract

Retinol and carotenoids were measured in Dutch milk and dairy products using a validated approach based on complete extraction of fat, followed by mild saponification and analysis by high-performance liquid chromatography. Raw milk, full fat milk, semi-skimmed milk and butter contain about 10 µg retinol and 6 g carotenoids per g fat. Values for retinol equivalents in milk are 10–20% higher than the values published in the Dutch food composition table. β-Carotene comprises 90% of total carotenoids present in cow’s milk, contrary to values published for human milk, which show more equally distributed carotenoids. Winter milk contains 20% less retinol and β-carotene compared to summer milk. Retention of retinol and β-carotene per g fat in hard cheese is one third to one half relative to the corresponding raw milk. In liquid and semi-liquid dairy products (pasteurized milk, buttermilk, vanilla custard, and yoghurt) retention of both compounds is above 80%. Recovery of carotenoids using the validated method is better than that reported by others previously.

Keywords: Retinol; Carotenoids; Milk; Dairy

1. Introduction

Data from the 1998 Dutch food consumption survey show that, in the Netherlands, average intake of vitamin A in all age groups ≥4 yr is below the recommended daily intake (Hulshof et al., 1998). The main sources of vitamin A in the Dutch diet are edible fats such as margarines, meat and meat products, fruits and vegetables (containing provitamin A carotenoids), and milk and dairy products. Milk and dairy products contribute 15–20% to total vitamin A intake, depending on the age group (Goldbohm et al., 1998; Hulshof et al., 1998). Intakes of vitamin A and other nutrients are based on the Dutch food composition database (NEVO, 1996, 2001). In this database, values for retinol equivalents in milk do not include seasonal variation and the presence of provitamin A carotenoids (Westenbrink, NEVO, personal communication). In milk both vitamin A and carotenoids are stored in fat globules (Jensen, 1995) and hence are present in a highly bioavailable form (Castenmiller and West, 1998). The carotenoid pattern in milk is species dependent: in human milk the contribution of the different carotenoids to the total carotenoid content is more equally distributed among the five main carotenoids (lutein, cryptoxanthin, α-carotene, β-carotene, lycopene) than in cow’s milk (Kim et al., 1990; Giuliano et al., 1994; Khachik et al., 1997). Evidence has accumulated over the past decades that carotenoids, besides their well-known function as vitamin A precursor of those with a β-ionone ring structure, have other valuable properties. These properties include their anti-oxidant functions (radical and singlet oxygen quenching), role in cell differentiation, precursor of nuclear receptor ligand, and induction of cell-communication (Russel, 1998). This
may be of special importance for the (premature) newborn who fully relies on milk as the sole source of energy and nutrients during the first half year of life (Goldman et al., 1990; Jewell et al., 2001).

Most reports regarding the carotenoid content of milk deal with human milk and colostrum (Kim et al., 1990; Patton et al., 1990; Giuliano et al., 1992; Giuliano et al., 1994; Khachik et al., 1997; Liu et al., 1998). Ollilainen et al. (1989) reported about carotenoids in Finnish dairy products collected in autumn and winter. Carotenoids other than β-carotene were only present in trace amounts. It is uncertain whether the reported low content of carotenoids in dairy is influenced by the analytical methodology. Simultaneous analysis of retinol and carotenoids in milk is paved with difficulties due to the labile nature of the compounds, low concentrations, and the need for saponification in order to remove fat and to hydrolyze retinyl esters and probably carotenoid esters. Low recoveries of carotenoids during sample cleanup have been observed frequently (Patton et al., 1990; Giuliano et al., 1992; Liu et al., 1998).

The purpose of this paper is to present a valid method for simultaneous determination of retinol and provitamin A carotenoids in milk, to update the retinol and carotenoid content of milk and dairy products in the Netherlands taking into account seasonal variation, and to explore the effect of processing of raw milk on the retention of retinol and carotenoids. The approach is based on a simultaneous measurement of retinol and carotenoids in a saponified extract using high performance liquid chromatography (analytical method 1). To validate this method, we analyzed Reference Material 8435 (NIST, Gaithersburg, USA), a spray-dried milk powder with reference concentrations for vitamin A (analytical method 2).

2. Materials and methods

2.1. Samples

All samples were collected by the Netherlands controlling authority for milk and milk products (COKZ), Leusden, the Netherlands, based on a mutually agreed protocol.

2.2. Effect of season

Milk: Raw milk, pasteurized full fat milk and pasteurized semi-skimmed milk were sampled at three Dutch dairy factories in Heiloo (province Noord-Holland), Maasdam (province Zuid-Holland), and in Nijkerk (province Gelderland). From each factory and during each season 3–4 samples of raw milk were taken. Pasteurized milk samples were compiled from 4 subsamples of 1 L each, taken within a 2-week period, and pooled to one sample. Each sub-sample was taken from a large batch of pasteurized milk (>10,000 L) prepared for distribution to retailers. Raw milk samples (0.5 L each) were taken from a batch (>10,000 L) of raw milk. All cooled samples (4 °C) were packed in polytetrafluoroethylene (PTFE) bottles, wrapped in aluminium foil and transported to COKZ at 4 °C. Samples were stored at −20 °C at COKZ until all samples for each 2 week period were collected. After each period, the samples were transported in frozen condition to the laboratory of the Division of Human Nutrition, Wageningen University.

Cheese: No carotene as colorant was added to the cheeses. Very young Gouda cheese was sampled from a dairy factory in Bleskensgraaf (province Zuid-Holland) in three seasons. Per season three 12-day-old cheeses were sampled. Each of the cheeses originated from the same batch of raw milk (sequence samples). Very young Edammer cheese was sampled from a dairy factory in Marum (province Friesland) and very young Maasdammer cheese was sampled from Steenderen (province Gelderland). From each type of cheese (Edammer or Maasdammer), three 12-day-old cheeses were sampled. Again, each of the three cheeses originated from the same batch of raw milk. One cheese from each type was packed in aluminium foil and stored at −20 °C until analysis. The two other cheeses from each type were subjected to a ripening process at NIZO food research (Ede, the Netherlands) at 13 °C and 85% relative humidity. The cheeses were ripened for the following times: young Gouda, 8 wk; mature Gouda, 24 wk; Edammer, 20 wk; and Maasdammer, 6 wk. After ripening, a 250 g section from each cheese was taken and stored at −20 °C until analysis.

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collection. Retinol and carotenoids in milk are stable for at least 60 days at −20 °C (Vidal-Valverde et al., 1992). This was confirmed by analysis of an internal control pool, in our laboratory.

2.4. Analytical methods: method 1, retinol and carotenoids

2.4.1. Chemicals and standards

Ethanol, petroleum ether (boiling point 40–60 °C), ammonia 25% (w/v), pyrogallol, isopropanol, anhydrous sodium sulfate, and potassium hydroxide were obtained from Merck (Darmstadt, Germany). Butylated hydroxy toluene (BHT) and triethylamine were obtained from Sigma Chemical Company (St Louis, MO, USA). Diethyl ether, methanol, tetrahydrofuran (THF) and dichloromethane were obtained from Labscan Limited (Dublin, Ireland). Hexane was obtained from Rathburn Chemicals Limited (Walkerburn, Scotland, UK). Methanol, tetrahydrofuran, isopropanol and dichloromethane were HPLC grade. α-Carotene, β-carotene, and lycopene standards were purchased from Sigma Chemical Company; zeaxanthin and β-cryptoxanthin were purchased from Roth (Karlsruhe, Germany). Retinol and lutein were purchased from Fluka (Buchs, Switzerland). Lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene from ampoules were initially dissolved in THF (containing BHT, 0.01% w/v). Then stock standard solutions of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene were prepared in either ethanol or hexane; stock standards of retinol were prepared in ethanol. Flasks containing the standards were placed in an ultrasound bath (Eurosonic 22, Wilten Woltil, De Meern, The Netherlands) for 10 min to ensure that the standards were dissolved completely. The concentrations of the standards were measured in a spectrophotometer (model M4 QIII, Carl Zeiss, Oberkochen/Württemberg, Germany), using the following extinction coefficients (E1%1cm) in ethanol: retinol, 1850 (λmax, 325 nm); lutein, 2550 (445 nm); and zeaxanthin, 2540 (450 nm). The extinction coefficients used for β-cryptoxanthin, all-trans-α-carotene, all-trans-β-carotene and lycopene in hexane were 2386 (452 nm), 2800 (444 nm), 2592 (453 nm) and 3450 (472 nm), respectively (De Ritter and Purcell, 1981). Purity of the individual standards was determined by HPLC. Final working standards were prepared in methanol/THF (3:1 v/v) after appropriate dilution of the stock standards. Working standards were shown to be stable for 6 weeks at −20 °C in the dark.

2.4.2. HPLC instrumentation

The HPLC system (Spectra, Thermo Separation Products Inc., San Jose, California, USA) was equipped with two pumps (model P4000), a solvent degasser (model SCM400), a temperature controlled auto sampler (model AS3000), a UV-visible forward optical scanning detector (model SM5000A), interface (model SN4000), and control and integration software (PC1000, version 2.5). A reversed phase Vydac 218TP53 column (250 × 3.2 mm ID) from The Separations Group (Heمسpera, CA, USA) was used, containing silica polymerically modified with C18 (300 Å pore diameter, 5 μm particle size). A Vydac guard column (model 218GD54, 10 × 4.6 mm ID), packed with material similar to the analytical column, was attached to the inlet of the analytical column. Separations were achieved using a mixture of methanol-water-THF-triethylamine (87.9: 10.0:2.0:0.1 v/v/v/v) for 0.25 min, followed by a linear gradient for 0.5 min, after which the methanol-tetrahydrofuran–triethylamine composition was maintained at 92.4:7.5:0.1 v/v/v. The column flow rate was 0.7 mL/min and total run time per sample was 20 min. Separations were monitored at 325 nm (0–5.75 min) and at 450 nm (5.75–20 min) for the identification and quantification of retinol and carotenoids, respectively (Hulshof et al., 1997).

2.4.3. Sample preparation (all samples were analysed in duplicate)

Milk and liquid dairy products. In a 10-mL glass culture tube with Teflon-coated screw cap were subsequently added and mixed: 1 mL thawed homogenized milk, 0.25 mL ammonia solution (25% w/v) and 1 mL ethanol (96% v/v). Fat was extracted from the mixture twice with 2 mL diethyl ether containing BHT (0.0025% w/v) and 2 mL petroleum ether and shaken on a horizontal reciprocal laboratory-shaking machine (model SM 25, Edmund Bühler, Hechingen, Germany) for 5 min at 250 reciprocations per minute. Upper layers were collected after centrifugation for 2 min at 3000 g and evaporated in a dry block heater at 35 °C under a nitrogen atmosphere. The dry residue was saponified by adding 1.5 mL potassium hydroxide (5% w/v in 96% v/v ethanol), containing pyrogallol (0.2% w/v). The tubes were purged with nitrogen, firmly closed, placed in the dark, and the content mixed for 3 h on a tube shaker at 200 reciprocations per minute. After addition of 1.5 mL water, the mixture was extracted twice with 3 mL hexane. The hexane layers were combined and evaporated in a dry block heater as described above. The residue was dissolved in 250 μL methanol-THF (3:1 v/v) and 25 μL was injected into the HPLC system.

Cheese: Cheese (1 g) was extracted with 30 mL tetrahydrofuran containing BHT (0.01% w/v) in a 100 mL measuring cylinder, using a rod mixer (Polytron PT 20 OD, Kriens/Luzern, Switzerland) at moderate speed for 1 min. Prior to each extraction, 2.0 g anhydrous sodium sulfate was added. The extract was filtered through paper (Whatman, No 54, diameter 11 cm). The residue was re-extracted until the extract was colourless (usually 4 extractions were required). Filtrates were
combined and evaporated on a rotary evaporator to a volume of approximately 25 mL, transferred to a volumetric flask of 50 mL and brought to volume with THF. Two millilitres was transferred into a 10 mL glass culture tube with Teflon-coated screw cap, evaporated to dryness, and saponified as described above.

2.5. Analytical methods: method 2, retinol (for validation purpose)

The method described by Elburg et al. (2003) was used as reference method for the determination of retinol in milk.

2.5.1. Chemicals and standards—as above

HPLC instrumentation: The same HPLC system as above was used, with the exception of column and mobile phase. A normal phase BDS Hypersil CN column (150 x 3.0 mm ID, 5 μm particle size) fitted with a Javelin NH2 guard column from Keystone Scientific (Bellefonte, USA) was used. The mobile phase was hexane containing 0.01% BHT/isopropanol (98.5:1.5 v/v) at a flow rate of 0.7 mL/min.

Sample preparation: In a 10 mL glass culture tube with Teflon-coated screw cap were subsequently added and mixed: 1.0 mL of homogenized milk or reconstituted milk powder (Reference Material 8435, NIST, Gaithersburg, USA), 1.25 mL 96% ethanol containing pyrogallol (0.04% w/v), and 0.25 mL potassium hydroxide solution (50% w/v in water). The tubes were purged with nitrogen, firmly closed and placed in a water bath of 80°C for 30 min. After cooling to room temperature, the mixture was extracted three times with 2.0 mL hexane containing BHT (0.01% w/v), using an Edmund Bühler SM 25 reciprocal laboratory shaker for 5 min at 250 reciprocations per minute. The hexane layers were removed, combined and mixed and then an aliquot was transferred to a HPLC vial from which 50 μL was injected into the HPLC system.

Moisture: Moisture was determined in all samples according to Osborne and Voogt (1978) by drying 1 g of sample in a vacuum oven at 70°C for at least 18 h until constant weight.

Fat: Fat was determined gravimetrically according to the Roese–Gottlieb method (Association of Official Analytical Chemists (AOAC), 1999 procedure 905.02).

Quality control: In each sample run, an in-house control sample (pasteurized full fat milk, “boerendam-melk”, purchased from Albert Heijn, Zaandam, The Netherlands) was analysed in duplicate to monitor stability of the measurement of retinol and carotenoids over time. Within-run and between-run coefficients of variation were 6.0% and 1.5% for retinol and 3.8% and 5.4% for β-carotene, respectively, over a 1-year period. Recoveries of standards added prior to the extraction of fat were 99 ± 11% for retinol, 74 ± 4% for lutein, 67 ± 3% for zeaxanthin, 111 ± 6% for β-cryptoxanthin, 98 ± 5% for x-carotene, 102 ± 12% for β-carotene, and 101 ± 13% for lycopene (n = 6 at intervals during the period of analysis). To assess method bias, the retinol concentration in Reference Material 8435 (spray dried whole milk powder) was measured using both methods. For the determination of fat, within-run and between-run coefficients of variation were 1.2% and 1.5%, respectively.

Differences between winter-spring milk and summer-autumn milk were analysed by ANOVA using SPSS (release 11.0.1, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Effect of season

Retinol and carotenoid content of raw and processed milk are presented in Table 1 (in μg/100 g) and in Table 2 (in μg/g fat). Raw milk (4.4% fat) contains on average 40 μg retinol and 20 μg carotenoids per 100 g. Full fat milk (standardized to 3.5% fat) and semi-skimmed milk (standardized to 1.5% fat) contain 34 and 14 μg/100 g retinol and 18 and 9 μg/100 g carotenoids, respectively. β-Carotene is the predominant carotenoid in dairy, comprising approximately 90% of total carotenoid content. A carotenoid profile of cow’s milk in which β-carotene dominates in similar amounts was also reported by Ollilainen et al. (1989). Milk sampled during winter and early spring (January–April) contains significantly less retinol and β-carotene (approximately 20% less) than milk sampled during summer and early autumn (July–October). This is independent of the fat content (Table 2). Seasonal differences in animal feeding practices may be the main cause for these differences in nutrient content: during summer and autumn, cows mainly stay in the pasture or when not, are fed freshly cut grass, in addition to ensilage and pellets containing grains, soy and tapioca. During winter and spring, cows stay in stables and are mainly fed with ensilage (from grass and corn) and pellets. We did not expect to find regional differences in retinol and carotenoid content in milk, due to the homogeneous climatological zone, cattle breed and animal feeding practices in the Netherlands. This was confirmed by retinol and carotenoid analysis of raw milk from three locations (data not shown). Assuming a bioconversion of β-carotene to retinol of 1:0.3 (FAO/WHO, 1988) or 1:0.5 (Institute of Medicine (IOM), 2001), it can be calculated that β-carotene contributes 20% to the vitamin A activity in milk according to the FAO/WHO conversion factors or 33% according to the IOM conversion factors. The vitamin A activity of full fat milk in this study, calculated as retinol equivalents (RE) per g fat equals 11.4 μg/g fat or 12.3 μg/g fat, depending on the conver-
## Table 1
Retinol and carotenoids in raw and processed milk

<table>
<thead>
<tr>
<th>Product</th>
<th>Sampling date</th>
<th>N</th>
<th>Fat (g/100 g (S.D.))</th>
<th>Dry matter (g/100 g (S.D.))</th>
<th>Retinol (µg/100 g (S.D.))</th>
<th>Lutein (µg/100 g (S.D.))</th>
<th>Zeaxanthin (µg/100 g (S.D.))</th>
<th>ß-Cryptoxanthin (µg/100 g (S.D.))</th>
<th>α-Carotene (µg/100 g (S.D.))</th>
<th>ß-Carotene (µg/100 g (S.D.))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>March 2000</td>
<td>10</td>
<td>4.5 (0.1)</td>
<td>13.6 (0.4)</td>
<td>39.6 (2.8)</td>
<td>0.8 (0.1)</td>
<td>0.1 (0.1)</td>
<td>0.4 (0.1)</td>
<td>&lt;0.1β</td>
<td>18.6 (2.1)</td>
</tr>
<tr>
<td></td>
<td>September 2000</td>
<td>10</td>
<td>4.2 (0.1)β</td>
<td>13.5 (0.1)</td>
<td>46.9 (3.3)β</td>
<td>1.6 (0.4)β</td>
<td>0.1 (&lt;0.1)</td>
<td>0.3 (0.1)</td>
<td>0.2 (0.1)</td>
<td>22.3 (2.5)β</td>
</tr>
<tr>
<td></td>
<td>April 2001</td>
<td>10</td>
<td>4.5 (0.2)</td>
<td>13.7 (0.2)</td>
<td>35.0 (1.9)</td>
<td>0.8 (0.1)</td>
<td>0.1 (&lt;0.1)</td>
<td>0.3 (0.1)</td>
<td>&lt;0.1</td>
<td>15.6 (1.8)</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>30</td>
<td>4.4 (0.2)</td>
<td>13.6 (0.4)</td>
<td>40.5 (3.6)</td>
<td>1.1 (0.4)</td>
<td>0.1 (0.1)</td>
<td>0.3 (0.1)</td>
<td>0.1 (0.1)</td>
<td>18.8 (3.5)</td>
</tr>
<tr>
<td>Pasteurized, full fat milk</td>
<td>October 1999</td>
<td>3</td>
<td>3.4 (&lt;0.1)</td>
<td>12.6 (0.3)</td>
<td>35.7 (0.2)β</td>
<td>1.2 (0.2)β</td>
<td>0.1 (&lt;0.1)</td>
<td>0.4 (0.1)</td>
<td>0.1 (&lt;0.1)</td>
<td>19.1 (2.1)β</td>
</tr>
<tr>
<td></td>
<td>February 2000</td>
<td>3</td>
<td>3.5 (0.1)</td>
<td>12.4 (0.4)</td>
<td>31.2 (2.4)</td>
<td>0.8 (0.1)</td>
<td>0.1 (&lt;0.1)</td>
<td>0.3 (0.1)</td>
<td>&lt;0.1</td>
<td>15.3 (0.9)</td>
</tr>
<tr>
<td></td>
<td>April 2000</td>
<td>3</td>
<td>3.5 (0.2)</td>
<td>12.4 (0.1)</td>
<td>30.9 (2.9)</td>
<td>0.8 (0.1)</td>
<td>&lt;0.1</td>
<td>0.3 (&lt;0.1)</td>
<td>&lt;0.1</td>
<td>14.7 (1.3)</td>
</tr>
<tr>
<td></td>
<td>July 2000</td>
<td>3</td>
<td>3.2 (0.2)</td>
<td>12.1 (0.4)</td>
<td>36.8 (3.6)β</td>
<td>1.4 (0.2)β</td>
<td>0.1 (0.1)</td>
<td>0.3 (&lt;0.1)</td>
<td>0.1 (0.1)</td>
<td>17.8 (1.4)β</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>12</td>
<td>3.4 (0.2)</td>
<td>12.4 (0.3)</td>
<td>33.6 (3.6)</td>
<td>1.0 (0.3)</td>
<td>0.1 (&lt;0.1)</td>
<td>0.3 (0.1)</td>
<td>0.1 (&lt;0.1)</td>
<td>16.7 (2.2)</td>
</tr>
<tr>
<td>Pasteurized, semi-skimmed milk</td>
<td>October 1999</td>
<td>3</td>
<td>1.5 (0.1)</td>
<td>10.6 (0.1)</td>
<td>15.9 (0.2)β</td>
<td>0.7 (0.1)β</td>
<td>0.1 (&lt;0.1)</td>
<td>0.1 (0.1)</td>
<td>&lt;0.1</td>
<td>9.4 (1.1)β</td>
</tr>
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<td></td>
<td>February 2000</td>
<td>3</td>
<td>1.5 (&lt;0.1)</td>
<td>10.5 (0.1)</td>
<td>12.9 (0.1)</td>
<td>0.5 (0.1)</td>
<td>&lt;0.1</td>
<td>0.1 (0.1)</td>
<td>&lt;0.1</td>
<td>7.0 (0.4)</td>
</tr>
<tr>
<td></td>
<td>April 2000</td>
<td>3</td>
<td>1.4 (0.1)</td>
<td>10.7 (0.2)</td>
<td>13.2 (1.6)</td>
<td>0.6 (0.2)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>6.8 (0.3)</td>
</tr>
<tr>
<td></td>
<td>July 2000</td>
<td>3</td>
<td>1.3 (0.2)</td>
<td>10.3 (0.3)</td>
<td>15.6 (0.1)β</td>
<td>0.8 (0.1)β</td>
<td>0.1 (&lt;0.1)</td>
<td>0.1 (&lt;0.1)</td>
<td>&lt;0.1</td>
<td>8.3 (0.8)β</td>
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<tr>
<td></td>
<td>Average</td>
<td>12</td>
<td>1.4 (0.1)</td>
<td>10.5 (0.2)</td>
<td>14.4 (1.6)</td>
<td>0.6 (0.2)</td>
<td>&lt;0.1</td>
<td>0.1 (0.1)</td>
<td>&lt;0.1</td>
<td>7.8 (1.3)</td>
</tr>
<tr>
<td>Butter</td>
<td>October 1999</td>
<td>2</td>
<td>80.8 (0.4)</td>
<td>—d</td>
<td>778.1 (30.6)</td>
<td>18.8 (1.8)</td>
<td>0.3 (0.4)</td>
<td>7.5 (0.7)</td>
<td>2.5 (1.8)</td>
<td>430.9 (31.4)β</td>
</tr>
<tr>
<td></td>
<td>January 2001</td>
<td>2</td>
<td>79.4 (0.4)</td>
<td>—d</td>
<td>733.5 (17.2)</td>
<td>14.5 (2.8)</td>
<td>&lt;0.1</td>
<td>7.1 (&lt;0.1)</td>
<td>&lt;0.1</td>
<td>295.5 (20.9)</td>
</tr>
<tr>
<td></td>
<td>July 2000</td>
<td>2</td>
<td>79.7 (0.1)</td>
<td>—d</td>
<td>887.5 (15.4)</td>
<td>25.9 (5.7)</td>
<td>2.4 (1.1)</td>
<td>5.4 (0.3)</td>
<td>2.2 (1.0)</td>
<td>384.2 (29.2)β</td>
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<tr>
<td></td>
<td>Average</td>
<td>6</td>
<td>80.0 (0.7)</td>
<td>—d</td>
<td>799.7 (72.9)</td>
<td>19.7 (5.9)</td>
<td>0.9 (1.3)</td>
<td>6.6 (1.1)</td>
<td>1.6 (1.5)</td>
<td>370.2 (65.0)</td>
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<tr>
<td>Young cheese, Gouda (8 weeks)</td>
<td>October 1999</td>
<td>3</td>
<td>30.7 (0.1)</td>
<td>60.5 (0.8)</td>
<td>124.7 (7.6)</td>
<td>4.1 (1.1)</td>
<td>0.2 (0.2)</td>
<td>0.1 (0.2)</td>
<td>&lt;0.1</td>
<td>61.5 (9.3)</td>
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<tr>
<td></td>
<td>April 2000, July 2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ripened cheese, Gouda (26 weeks)</td>
<td>October 1999, July 2000</td>
<td>2</td>
<td>30.2 (2.3)</td>
<td>62.6 (1.4)</td>
<td>112.1 (13.3)</td>
<td>2.9 (0.2)</td>
<td>0.2 (0.1)</td>
<td>0.2 (0.3)</td>
<td>&lt;0.1</td>
<td>47.8 (7.0)</td>
</tr>
</tbody>
</table>

* Values <0.1 µg/100 g are below limit of quantitation.
* Significantly different from winter/spring (January–April) samples ($P < 0.05$). Comparisons are within the same product.
* Each sample was compiled by pooling 4 sub-samples of 1 L each within a 2-week period.
* Not determined.
The factor used for β-carotene. This is slightly higher than the values reported by the Dutch (10.0 μg/g fat), Finnish (9.5 μg/g fat), USDA (9.5 μg/g fat), German (8.6 μg/g fat), and New Zealand (5.3 μg/g fat) nutrient databases, but lower than the UK (14.4 μg/g fat) and South African (16.2 μg/g fat) nutrient databases (NEVO, 1996, 2001; Rastas et al., 1997; US Department of Agriculture, Agricultural Research Service, 2001; Souci et al., 1994; Burlingame et al., 1994; Holland et al., 1991; Smit et al., 1998). Most plausible is that these differences are due to sampling variation, analytical methods, differences in inclusion of carotenoids in the calculation of retinol equivalents, differences in cattle breed, and differences in cattle feeding practices. Milk produced in the Netherlands is mainly from Holstein–Friesian cows.

### 3.2. Effects of processing

Differences in retinol and carotenoid content between young (8 wk ripening) and mature Gouda cheese (26 wk ripening) were small: 125 ± 7 and 112 ± 13 μg retinol/100 g, and 62 ± 9 and 48 ± 7 μg β-carotene/100 g respectively (Table 1 and 2, Fig. 1), indicating that ripening does not result in loss of retinol or β-carotene. Although only two mature and three young cheeses were analysed, the cheeses were processed from the same batch of raw milk and ripened under strictly controlled conditions. Per g fat, less than 50% retinol and β-carotene is retained in the cheese relative to raw milk. It seems that the main losses of retinol and β-carotene in Gouda cheese occur during the first 12 days in the cheese making process (Fig. 1). Retention of retinol and β-carotene in the fat fraction of 12-day-old Gouda cheese is only 46 ± 4% and 38 ± 3%, respectively relative to the corresponding raw milk. In contrast, retention of retinol and β-carotene in 12-day-old Edammer cheese is 81% and 70%, respectively (Fig. 1). However further ripening of Edammer cheese seems to diminish this divergence and leads to a similar retention of both retinol and β-carotene of about 40%. Retention of retinol and β-carotene of Maasdammer cheese is comparable to Gouda cheese of similar age.

Retention of retinol in liquid and semi-liquid dairy products range from 83% for buttermilk to 98% for full fat milk, 3.4% fat (Fig. 1). For β-carotene this ranged from 92% for yoghurt to 100% for milk and buttermilk. Contrary to the hard cheeses, where retention of β-carotene seems to be slightly lower than retention of retinol, there is a tendency for higher retention of β-carotene in liquid and semi-liquid dairy products.

### 3.3. Effect of analysis

Table 3 presents results of analysis of Reference Material 8435, prepared from spray-processed whole milk powder. The mild extraction and saponification
method (method 1, used in this study) recovered only approximately 50% of the retinol in the Reference Material. However method 2 (hot saponification of the milk sample) recovered all retinol present in the Reference Material. Since method 1 and method 2 gave similar results in a common milk matrix (Table 3), our method is valid for the determination of retinol in liquid milk. No reference values are available for carotenoids in the Reference Material. We did not use the hot saponification procedure (method 2) for the milk samples in this study since we encountered losses of \( \beta \)-carotene up to 10% during direct hot saponification in a watery matrix and suspected that it would be even worse for other carotenoids (Patton et al., 1990; Giuliano et al., 1992; Liu et al., 1998). Kim et al. (1990) reported recoveries of 93% and 97% for retinol and \( \beta \)-carotene respectively in human milk using a hot (75°C) saponification procedure with a final KOH concentration of 6% (w/v) in the saponification mixture. No information on recovery of other carotenoids was reported. Patton et al. (1990) saponified human colostrum for 1 h at 45°C in a final KOH concentration of 17% (w/v). Recoveries of 90–100% were claimed for all carotenoids, except for lutein and zeaxanthin (approximately 50%). Giuliano et al. (1992) saponified human milk in a watery matrix for 0.5 or 16 h, in a final KOH concentration of 13% (w/v), at different temperatures. Losses upon saponification were temperature dependent and highest for lycopene (up to 68%) and \( \beta \)-carotene (>40%). Liu et al. (1998) used both enzymatic saponification (protease and lipase treatment) and chemical saponification (final KOH concentration 17% w/v) of human milk in a watery matrix at 37°C for 30 min. Recoveries above 80% were reported except for lutein/zeaxanthin (unresolved peak), which was 61%. We found, in agreement with Patton et al. (1990) and Liu et al. (1998), lower recoveries for lutein and zeaxanthin. In our study this was on average 67% for zeaxanthin and 74% for lutein. We have no explanation for the lower recovery of the dihydroxy xanthophylls lutein and zeaxanthin. Generally carotenoids are stable upon alkaline saponification, except for some specific compounds with a keto group (Britton et al., 1995).

For analysis of human milk samples this is of more concern than for analysis of cow’s milk, since in human milk the contribution of different carotenoids to the total carotenoid content is more equally distributed among the 5 main carotenoids: lutein, cryptoxanthin, \( \alpha \)-carotene, \( \beta \)-carotene, and lycopene, including isomers (Kim et al., 1990; Giuliano et al., 1994; Khachik et al., 1997). Our approach in the analysis of carotenoids is based on mild saponification of the fat extract (final

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**Table 3**

Retinol content in Reference Material 8435 (whole milk powder) and in full-fat milk, assessed by mild saponification of the fat extract (method 1) and by hot saponification of milk (method 2)

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>RM 8435(^a)</th>
<th>Full fat milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/100 g (S.D.)(^b)</td>
<td>µg/100 g (S.D.)(^b)</td>
</tr>
<tr>
<td>Method 1</td>
<td>90 (31)</td>
<td>35.1 (0.2)</td>
</tr>
<tr>
<td>Method 2</td>
<td>189 (2)</td>
<td>34.5 (0.1)</td>
</tr>
</tbody>
</table>

\(^a\)Reference concentration: 190µg/100 g.

\(^b\)Triplicate analysis.

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**Fig. 1.** Retention of retinol and \( \beta \)-carotene (% per g fat) in dairy, after processing of corresponding raw milk. Retention in raw milk is set at 100%.

- a = Gouda cheese, not ripened (12 days); retention (n = 3): retinol 46 ± 4%, \( \beta \)-carotene 38 ± 3%
- b = Gouda cheese, ripened (8 wk); retention (n = 3): retinol 42 ± 8%, \( \beta \)-carotene 39 ± 3%
- c = Gouda cheese, ripened (26 wk); retention (n = 2): retinol 34 ± 1%, \( \beta \)-carotene 29 ± 5%
- d = Edammer cheese, not ripened (12 days); e = Edammer cheese, ripened (20 wk); f = Maasdammer cheese, not ripened (12 days);
- g = Maasdammer cheese, ripened (6 wk); h = full cream milk, 3.4% fat;
- i = low fat milk, 1.4% fat;
- j = full cream milk, 4.4% fat;
- k = buttermilk;
- l = vanilla custard;
- m = yogurt;
- \( \text{---} \) = retinol;
- \( \text{---} \) = \( \beta \)-carotene.
KOH concentration 5%) in a non-watery environment, in order to avoid conversion of β-carotene into monooepoxy carotenes and trans–cis isomerization (Kimura et al., 1990). This approach has also been proven to be fruitful in the analysis of carotenoids in fat aspirates, and for the analysis of human milk samples from Indonesian populations (Dijkstra, M.A., Wieringa, F.T., West, C.E., Muherdiyantiningsih, R., 1998. Bioavailability and bioconversion of liquid and semi-liquid dairy products leads to losses of retinol and β-carotene up to almost 20%. In mature hard cheeses such as Gouda cheese, only one third to half of the retinol initially present in raw milk is retained.

Acknowledgements

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References


