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Short communication

Determination of liposoluble vitamins in cooked meals, milk and milk products by liquid chromatography ☆

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Abstract

A method for the simultaneous determination of liposoluble vitamins in cooked meals was established. Saponification was performed with 50% (w/v) KOH at 80 °C, and ascorbic acid was added as antioxidant. The subsequent extraction was carried out with diethyl ether. This was followed by a liquid chromatographic separation on a reversed-phase C_{18} column with methanol–water (94:6, v/v) as the mobile phase. Retinyl acetate was used as the internal standard. The analytical parameters linearity, detection limit (0.19 and 8.33 μg/100 g for retinol and α-tocopherol, respectively), precision of the method (RSD=5.24 and 6.99% for retinol and α-tocopherol, respectively) and recovery assays (95.6 and 96.5% for retinol and α-tocopherol, respectively) show that the method studied is useful for measuring these compounds in foods and cooked meals.

Keywords: Food analysis; Milk; Vitamins; Retinol; Tocopherols

1. Introduction

The main lipophilic antioxidant is α-tocopherol, which inhibits peroxidation of polyunsaturated fatty acids in cell membranes. It is a liposoluble vitamin with an antioxidant capacity, reacting with peroxy radicals and other free radicals [1,2]. High concentrations of antioxidants, including α-tocopherol, are associated with a reduction in the risk of disorders connected to free radicals, such as atherosclerosis, cancer, cataracts and cell damage connected to ischaemia and reperfusion. Reduction in the risk of coronary illness as a result of a high intake of vitamin E has also been indicated [3–8]. The most effective way of fighting cardiovascular illnesses caused by free radicals is prevention, and in this respect diet can play an important part [9–15]. Numerous studies have linked intake of vitamins A and E with prevention of cancer [16–19].

Liposoluble vitamins are provided by food intake, and the content of these vitamins will vary in relation to the foods of which it consists and the treatments to which they are subjected. The stability of vitamin A varies according to the food selected and the way in which it is prepared and/or stored. Vitamin A can be quite unstable when the pH is reduced to 4.5 or

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lower. Low pH will cause both isomerization of all-*trans*-vitamin A to the less potent cis forms, and de-esterification of vitamin A esters to the more-labile retinol [20]. This makes it very difficult to predict the exact vitamin content of foods. Vitamin E is found in foods in four main forms, α-, β-, γ- and δ-tocopherols. In most foods, α-tocopherol is more variable than one might expect, depending on the origin, preparation, and storage temperature and age of the food product [21].

The technique most frequently used now for simultaneous determination of liposoluble vitamins is liquid chromatography [22–32].

The aim of the present study was to establish a method for the simultaneous determination of liposoluble vitamins (vitamins A and E) in milk and milk products and in ready-to-eat meals, and to compare the values obtained with those given in food composition tables.

2. Experimental

2.1. Chemicals

Only analytical-grade substances were used: potassium hydroxide, phenolphthalein, sodium sulphate, and diethyl ether from Panreac Química (Barcelona, Spain) and absolute ethanol and methanol (HPLC grade) from Mallinckrodt Baker (Deventer, Netherlands). Distilled-deionized water was from a Millipore Milli-Q water system (Millipore, Jafrey, MA, USA). Ergocalciferol, cholecalciferol, all-*trans*-retinol and α-tocopherol were from Sigma (Taufkirchen, Germany), retinyl acetate from Fluka (Buchs, Switzerland) and ascorbic acid from Merck (Darmstadt, Germany). All solvents used in LC determinations were filtered through 0.22-μm pore size filters.

The retinyl acetate, retinol and α-tocopherol stock solutions (1 mg/100 ml) were prepared in absolute ethanol and stored at 4 °C in light-resistant glass bottles. The working solutions were prepared daily.

2.2. Equipment

The LC system consisted of a series 1050 chromatograph with a quaternary pump system, a UV detector, an on-line degassing system, and a ChemStation (series MS-DOS, 3365) integrator (Hewlett-Packard, Waldbronn, Germany).

The filters, with a pore size of 0.22 μm and diameter of 47 mm, were from Magna Nylon, MSI (Micron Separations, Westborough, MA, USA).

2.3. Column

A Spherisorb ODS-2 C_{18} column, with a particle size of 5 μm, 250×4.6 mm (Teknokroma, Barcelona, Spain), was used.

2.4. Sample preparation

Individual lunch and dinner servings were collected daily for six consecutive days (from Monday to Saturday; Table 1), in triplicate and weighed after inedible fractions had been removed. They were homogenized jointly (Polytron PT/2000 homogenizer, Kinematica, Littau, Switzerland). The homogenized result was placed in polyethylene bags and stored at −18 °C until analysis.

In view of the wide variety of choice for breakfast, milk, margarine, cheese and yoghurt were analyzed, these being the foods that provide the greatest contribution of the vitamins studied.

2.5. Mobile phase

The mobile phases most commonly used are mixtures of methanol–water and methanol–acetonitrile. Tests were therefore made with the following mobile phases in various proportions: water–methanol, methanol, acetonitrile–methanol, acetonitrile and water–acetonitrile. The tests were carried out with standard solutions. It was observed that methanol either alone or mixed with acetonitrile did not achieve resolution of peaks, and that the use of pure acetonitrile or acetonitrile with increasing percentages of water lengthened the retention time of α-tocopherol, which in turn prolonged the duration of the analysis. Therefore, methanol was assayed with varying percentages of water. The best peak separation was obtained with a methanol–water (96:4, v/v) mixture. The retention times obtained were 4.01±0.11, 11.56±0.15, 12.4±0.09 and 16.00±0.13 min for retinol, ergocalciferol, cholecalciferol and

Table 1
Individual lunch and dinner servings collected daily for six consecutive days (from Monday to Saturday)

Day	Lunch	Dinner
1	Vegetable soup, macaroni, hamburger with fried vegetables, strawberries with cream, bread	Salad, sole in breadcrumbs, mayonnaise, cold meat, pineapple mousse, bread
2	Cold meat, escalope with cheese filling with salad, rich stew of chickpea, watermelon, bread	Vegetable pureé, roast chicken and chips, salad with chopped, fruit salad, bread
3	Salad, rice with chard, squid in breadcrumbs, orange, bread	Fish soup, beef steak, artichoke, cheese, apple, bread
4	Rich stew of beans, pork, etc., fried egg, chopped, lemon juice, watermelon, bread	Stewed vegetables, roast chicken with roasted vegetables, cheese, caramel cream, bread
5	Paella, bonito grilled, pepper fried, tomato juice, caramel cream, bread	Artichoke sauté with potatoes, pork steak, salad, salami, orange, bread
6	Gazpacho (Andalusian cold soup), roast chicken, creamed potatoes, cold meat, hake in breadcrumbs, yoghurt, bread	Noodle soup, ham omelette, sardines in oil, peach, bread

α-tocopherol, respectively, $n=10$ in all cases. The internal standard used was retinyl acetate, which eluted at 6 min and was not present in the sample. Although it was possible to detect them simultaneously at 280 nm, the wavelength was varied in order to increase the sensitivity and selectivity of the detection. The wavelength at which the response was greatest for the various items was 325 nm for retinol and retinyl acetate, 265 nm for ergocalciferol and cholecalciferol, and 294 nm for α-tocopherol.

It is possible to determine the four vitamins simultaneously by means of a single injection in the chromatograph when working with foods that are rich in or enriched with vitamin D, such as milk and milk products, although in practice it is preferable to determine vitamins A and E in one aliquot and concentrate the volume in another aliquot for determination of vitamin D, which is found in a very small proportion in the sample. In the present study, therefore, we concentrated on determining vitamins A and E in cooked meals, leaving vitamin D for subsequent studies.

2.6. Saponification

A modification of the method proposed by the AOAC [33] was used for the determination of vitamin A. The vitamins being studied are sensitive to oxidation, and retinol is also sensitive to ultraviolet light. Ascorbic acid was therefore added as an antioxidant, and saponification was performed in a nitrogen atmosphere protected from light. Various saponification conditions were also tested: at ambient temperature overnight, but it was found that saponification was not complete; at 37 °C for 15 h with agitation, which produced complete saponification, but emulsions that were difficult to break formed during the extraction. The conditions finally selected for saponification were: 10 g of sample was weighed, and 40 ml of absolute ethanol and 10 ml of 50% (w/v) KOH were added. The mixture was kept at 80 °C for 30 min, in a flow of inert gas (N_2) and with continuous agitation. In order to avoid possible oxidation, 0.3 g of ascorbic acid was added to the flask.

2.7. Extraction

Hexane is widely used for the determination of retinol, but Stancher and Zonta [34] studied the efficiency of hexane and diethyl ether for the determination of α-tocopherol and obtained the follow-

ing recoveries: 14.1±0.7% with hexane (4×50 ml), and 89.8±1.4% with diethyl ether (1×200 ml). Consequently, diethyl ether was chosen for the present study, and the extraction was performed with aliquots of 50 ml (four times).

When the unsaponifiable fraction had cooled, it was transferred to a decanting funnel, and the extraction was performed with 4×50 ml of diethyl ether (we verified that if the extraction was performed five times the result did not vary). The ether fractions were combined and washed with distilled water. The first liquid obtained from this process was re-extracted with diethyl ether, and the resulting extract was added to the ether phase. This was washed with distilled water until neutral to phenolphthalein. The ether extract was dried with anhydrous Na_2SO_4, collected in a 250-ml flask, and evaporated in a rotary evaporator (40 °C). The residue was collected with 1 ml of the internal standard solution (retinyl acetate in absolute ethanol, 1.6 μg/ml).

The samples were filtered through filters with a pore size of 0.22 μm and injected into the chromatograph (injection volume 20 μl). Quantification was performed by the internal standard method. Peak areas were used for quantitative analysis. Fig. 1 shows a chromatogram of a mixture of standards, and Fig. 2 shows a chromatogram of a combined lunch and dinner.

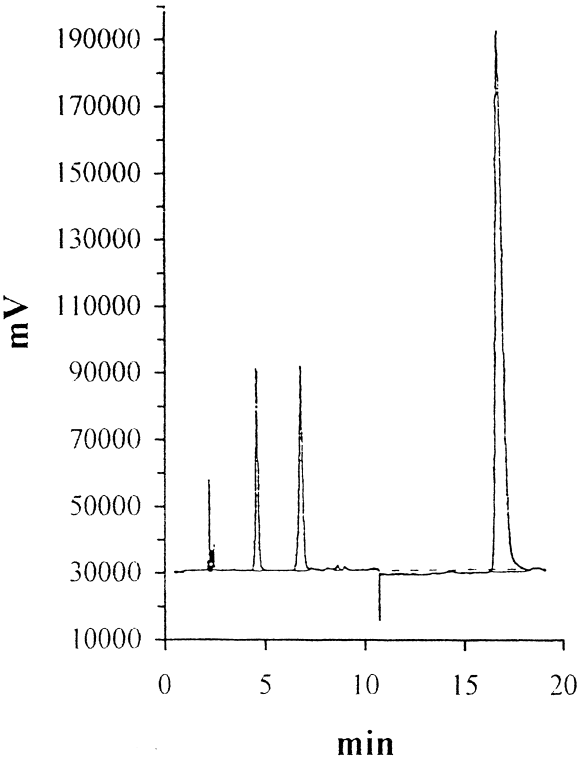

Fig. 1. Chromatogram of standards: (1) retinol (1.6 μg/ml), t_R=4.103 min; (2) retinyl acetate (1.6 μg/ml), t_R=6.35 min; (3) α-tocopherol (26.6 μg/ml), t_R=16.69 min. When the time is 0 the wavelength is 325 nm; it is changed to 294 nm at time 9.8 min.

3. Results

To check the reliability and usefulness of the proposed method the analytical parameters were determined.

The detection limits (calculated using signal/noise ratio) were 0.189 and 8.333 μg/100 g for retinol and α-tocopherol, respectively.

The responses were linear in the following ranges: 0.04–3.73 μg/ml (n=20) with a slope of 1.22 mV ml/μg (SD, 0.012 mV ml/μg), an intercept of 0.04 mV (SD, 0.019 mV), a standard error of 0.0577 and a correlation coefficient of 0.998 for retinol, and 5.5–550 μg/ml (n=20) with a slope of 0.02 mV ml/μg (SD, 0.0002 mV ml/μg), an intercept of 0.06 mV (SD, 0.031 mV), a standard error of 0.1668 and a correlation coefficient of 0.999 for α-tocopherol.

Instrumental precision was checked from six consecutive injections of a sample solution, and the relative standard deviations (RSDs) obtained were 3.33% (3.86±0.13 μg/100 g) and 2.98% (1019.26±30.37 μg/100 g) for retinol and α-tocopherol, respectively. The repeatabilities of the method (RSDs), checked from six batches of a sample, were 5.24% (15.81±0.83 μg/100 g) and 6.99% (933.47±65.25 μg/100 g) for retinol and α-tocopherol, respectively.

Accuracy was estimated by means of recovery assays. A sample (11.24 and 711.10 μg/100 g for retinol and α-tocopherol, respectively) to which known amounts of standard retinol and α-tocopherol (43.01 and 3746.17 μg/100 g, respectively) had been added was subjected to the entire extraction and determination process. The recovery percentages obtained were 95.6 and 96.5% for retinol and α-tocopherol, respectively.

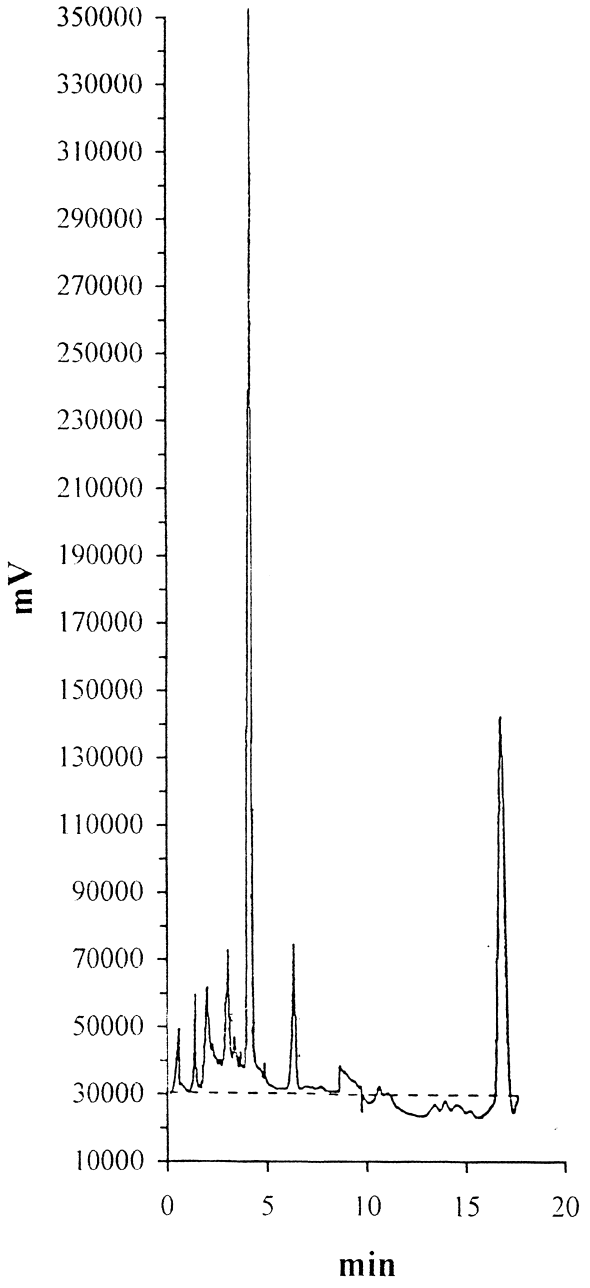

Fig. 2. Chromatogram of a combined lunch and dinner: (1) retinol, t_R=4.08 min; (2) retinyl acetate, t_R=6.33 min; (3) α-tocopherol, t_R=16.69 min. When the time is 0 the wavelength is 325 nm; it is changed to 294 nm at time 9.8 min.

Table 2
Retinol and α-tocopherol content (μg/100 g) in milk and milk products (breakfast items)

	Retinol (X±SD)		α-Tocopherol (X±SD)	
	Experimental	Tables	Experimental	Tables
Milk	28.67±3.38	30	93.89±15.68	88
Cheese	98.72±7.20	150	180.41±18.60	230
Yoghurt	17.26±2.94	13	53.03±6.50	37
Margarine	7.90±0.74	–	286.57±2.31	–

X, mean; SD, standard deviation.

4. Discussion

The values obtained in the determination of the analytical parameters show that this method is useful for measuring retinol and α-tocopherol.

Tables 2 and 3 show the results obtained when the method was applied with milk and milk products (cheese, yoghurt and margarine), which formed part of the breakfast and the complete servings analyzed for six consecutive days. If they are compared with the contents calculated from the values indicated in the food composition tables [35], it can be seen that in the case of retinol, the contents found experimentally are less than those calculated, whereas in the case of α-tocopherol the opposite occurs in the case of cooked meal. For cheese, the contents of the two vitamins found experimentally are less than those calculated, while in the case of milk and yoghurt the results obtained practically coincide with the information given by the food composition tables (Tables 2 and 3). In order to ascertain the vitamin contribution provided by a meal or a particular food, therefore, analytical determination is indispensable,

Table 3
Retinol (μg/day) and α-tocopherol (mg/day) content in cooked meals

Day	Retinol (X±SD)		α-Tocopherol (X±SD)	
	Experimental	Tables	Experimental	Tables
1	233.1±19.2	573.0	11.86±1.08	2.33
2	107.4±6.1	137.5	10.78±0.63	9.94
3	201.6±30.7	378.3	11.90±0.83	4.95
4	158.7±15.6	393.1	29.28±1.12	10.43
5	164.1±14.0	194.8	14.16±1.11	5.77
6	292.9±15.7	150.3	44.45±3.22	6.33

X, mean; SD, standard deviation.

since they are sensitive to the various processes to which they are subjected during manufacture, conservation and storage.

References

[1] P. Di Mascio, M. Murphy, H. Sies, Am. J. Clin. Nutr. 53 (1991) 194.
[2] H. Sies, W. Stahl, Am J. Clin. Nutr. 62 (1995) 1315s.
[3] M. Brown, E.J. Underwood, M.F. Ryan, Am. J. Clin. Nutr. 60 (1994) 383.
[4] S.N. Meydani, Am. J. Clin. Nutr. 60 (1994) 704.
[5] D.R. Janero, Crit. Rev. Food Sci. Nutr. 35 (1995) 65.
[6] P.B. Duell, J. Nutr. 126 (1996) 1067s.
[7] G. van Poppel, H. van den Berg, Cancer Lett. 114 (1997) 195.
[8] W.L. Scheider, L.A. Hershey, J.E. Vena, T. Holmlund, J.R. Marshall, Mov. Disord. 12 (1997) 190.
[9] A.B. Caragay, Food Technol. 46 (1992) 65.
[10] H.M.G. Princen, G. van Poppel, C. Vogelezang, R. Buytenhek, F.J. Kok, Arterioscler. Thromb. 12 (1992) 554.
[11] P.D. Reaven, A. Khouw, N.F. Beltz, S. Parthasarathy, J.L. Wiztum, Arterioscler. Thromb. 13 (1993) 590.
[12] M. Ferraroni, C. La Vecchia, B. D'Avanzo, E. Negri, F. Franceschi, A. Decarli, Br. J. Cancer 70 (1994) 1150.
[13] P. Six, Inform 5 (1994) 679.
[14] L.J. Machlin, Crit. Rev. Food Sci. Nutr. 35 (1995) 41.
[15] A. Ghisselli, A. D'Amicis, A. Giacosa, Eur. J. Cancer Prev. 6 (1997) 15s.
[16] R.R. Watson, T.K. Leonard, J. Am. Diet Assoc. 86 (1986) 505.
[17] D.I. Thurnham, J. Micronutr. Anal. 7 (1990) 279.
[18] J. Virtamo, Proc. Nutr. Soc. 58 (1999) 329.
[19] A.A. Botterweck, P.A. van den Brandt, R.A. Goldbohm, Cancer 88 (2000) 737.
[20] J.W. Erdman, C.L. Poor, J.M. Dietz, Food Technol. 42 (1988) 214.
[21] M.B. Reddy, M. Love, Adv. Exp. Med. Biol. 459 (1999) 99.
[22] W. Kneifel, F. Ulberth, U. Winkler-Macheiner, Dtsch. Lebensm. Rundsch. 83 (1987) 137.
[23] W.T. Wahyuni, K. Jinno, J. Micronutr. Anal. 3 (1987) 47.
[24] N. Bilic, R. Sieber, Z. Lebensm.-Unters.-Forsch. 186 (1988) 514.
[25] M.M. Delgado, A. Sanchez, M.C. Gomez, Analyst 120 (1995) 2489.
[26] B.Y. Gong, J.W. Ho, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 2389.
[27] M. Ake, H. Fabre, A.K. Malan, B. Mandrou, J. Chromatogr. A 826 (1998) 183.
[28] H. Qian, M. Sheng, J. Chromatogr. A 825 (1998) 127.
[29] M.J. Gonzalez, M. Tortras, A.I. Castellote, M.C. Lopez, Food Chem. 66 (1999) 221.
[30] J. Lee, K. Suknark, Y. Kluvitse, R.D. Phillips, R.R. Eitenmiller, J. Food Sci. 64 (1999) 968.
[31] D. Blanco, M.P. Fernandez, M.D. Gutierrez, Analyst 125 (2000) 427.
[32] W.O. Song, G.R. Becher, R.R. Eitenmiller, Modern Analytical Methodologies in Fat- and Water-soluble Vitamins (Chemical Analysis Series, Vol. 154), Wiley, New York, 2000.
[33] Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Washington, DC, 1990.
[34] B. Stancher, F. Zonta, Riv. Ital. Sostanze Grasse 60 (1983) 371.
[35] S.W. Souci, W. Fachmann, H. Krant, Food Composition and Nutrition Tables, Wissenschaftliche, Stuttgart, 1989.