



TRUEFOOD

Traditional United Europe Food

Contract no. FOOD-CT-2006-016264

Instrument: Integrated Project

Thematic Priority: Food Quality and Safety (# 5)

D4.1.2 -2

Initial title: Interim report including the results of the ring test.
New title: Interim report: analytical procedures selected for the analyses of milk carotenoids, vitamins A and E, fatty acids and volatile organic compounds

Due date of deliverable: April 2008

Actual submission date: April 2008

Start date of project: 1 May 2006

Duration: 48 months

Organisation name of lead contractor for this deliverable: IRTA – P10

Revision: final

Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	x
PP	Restricted to other programme participants (including the Commission Services)	
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**Initial title: Interim report including the results of the ring test.
New title: Interim report: analytical procedures selected for the analyses of milk carotenoids, vitamins A and E, fatty acids and volatile organic compounds.**

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Preliminary remark: *The purpose of this deliverable was first to report the results of the ring test we had planned between laboratories in order to homogenise the analytical procedures for the analyses of fatty acids, carotenoids and vitamins A and E in milks. However as the ring test has been dropped out, this report has been focused on the analytical procedures chosen for each milk compound analysis. Indeed, we first proposed that each partner analyses his own samples but finally, during the last technical meeting all the partners agreed that, in order to avoid any “laboratory effect”, the best solution was to analyse all the 375 samples in the same laboratory, chosen among the partners according to their own expertise. In particular, UMB (Norway) will analyse the milk carotenoids and vitamin A and E and INRA (France) will analyse the fatty acid profile of the 375 milks collected in the 4 countries. INRA will also perform all the analyses of volatile organic compounds.*

ABSTRACT

The aims of subtask 4.1.2 are (i) to describe the variability in the nutritional composition of tanker milks sampled in diversified situations in Europe and (ii) to link the variability in the milk composition to the production conditions.

The work in this on-farm experiment is achieved in parallel in Slovenia, Slovakia, Norway and France. In each country, from 15 to 20 farms or groups of farms have been selected in different locations with specific natural agro-climatic conditions and localization (distance from important human activities). The aim is to cover a wide range of situations that can be encountered in Europe. The milk samples will be collected in each farm or group of farms, 5 times in the course of year 2008. In total, 375 milk samples will be collected. The analyses will be made once all the milk samples have been collected.

The aim of this deliverable is to report the detailed analytical procedures chosen for the analyses of milk carotenoids, vitamins A and E, fatty acids and volatile organic compounds.

Introduction

The nutritional composition of dairy foods depends on a number of factors linked both to the composition of the raw material used and the subsequent processing. Milk nutritional composition is itself dependent on the milk production conditions that have increasingly been the focus of consumers' concern. In the case of Traditional Dairy Foods, milk nutritional composition is of special importance since any raw material modification during processing is restricted or prohibited. In Europe, the milk used by the dairy industry for traditional dairy foods is produced by cows reared over a very large variety of local conditions that can be responsible for a wide variability of the milk components with nutritional interest. Under experimental conditions, the latter have been shown to be dependant on the upstream factors like genetic (breeds), physiological (stage of lactation) and overall dietary factors. The 'natural' variability of the composition of the milks used by the dairies over Europe is not well known though it could be used by the dairies to sell dairy products that differ in components with a nutritional interest.

The aims of subtask 4.1.2 are to 1/ characterize the nutritional composition of tanker milk used by the dairy in France, Norway, Slovakia and Slovenia and 2/ identify the milk production conditions leading to milk naturally rich in interesting compounds that could provide added value to breeders and industry.

In particular, we will (i) describe the variability in the composition for fatty acids, carotenoids, vitamins A and E of tanker milks (corresponding to the mixture of several herds) sampled in diversified situations in Europe (Mediterranean, Continental, Alpine, Oceanic and Artic conditions), (ii) link the variability in the milk composition to the production conditions on the farms (mainly cow management and feeding).

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encountered in Europe. The milk samples will be collected in each farm or group of farms, 5 times in the course of year 2008. In total, 375 milk samples will be collected. The analyses will be made once all the milk samples have been collected.

The purpose of this deliverable was first to report the results of the ring test we had planned between laboratories in order to homogenise the analytical procedures for the analyses of fatty acids, carotenoids and vitamins A and E in milks. However as the ring test has been dropped out, this report has been focused on the analytical procedures chosen for each milk compound analysis. Indeed, we first proposed that each partner analyses his own samples but finally, during the last technical meeting all the partners agreed that, in order to avoid any “laboratory effect”, the best solution was to analyse all the 375 samples in the same laboratories, chosen among the partners according to their own expertise. In particular, UMB (Norway) will analyse the milk carotenoids and vitamin A and E and INRA (France) will analyse the fatty acid profile of the 375 milks collected in the 4 countries. In addition, INRA will also focus on other components with nutritional (phenolic compounds) or “anti-nutritional” properties (volatile organic compounds including pollutants like benzene derivatives or halogenated compounds).

The detailed analytical procedures chosen for the analyses of milk carotenoids, vitamins A and E, fatty acids and volatile organic compounds are described in this deliverable. The analytical procedure that will be used for the analysis of phenolic compounds is not yet stated.

Analysis of Carotenoids and Vitamins A and E in milk

1. Determination of Vitamin A in milk

Two parts milk will be hydrolyzed with three parts 12.5 M KOH/ethanol (2:1 v/v) for 25 min at 80 °C. BHT will be added as an antioxidant. After cooling, retinol will be extracted with hexane/toluene (1:1 v/v). The extract will then be submitted to chromatographic analysis on a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP1100 fluorescence detector, em: 325 nm ex: 480 nm. Vitamin A compounds will be separated on a 4.6 mm x 100 mm normal phase silica column using 2 % 2-propanol in hexane as the mobile phase. A three-point calibration curve will be used for quantification.

2. Determination of tocopherols in cow milk

One mL of milk will be diluted with 3 mL 2-propanol containing the internal standard tocol and BHT as an antioxidant. After thorough mixing (15 min) and centrifugation (10 min, 4000 g at 10 °C), an aliquot of 20 µL will be injected from the supernatant into the HPLC system. HPLC will be performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP1100 fluorescence detector, em: 295 nm ex: 330 nm. Tocopherol isomers will be separated on a 2.1 mm x 250 mm reversed phase column. A two-point calibration curve will be made from analysis of a 3 % albumin solution enriched with known concentration of tocopherols.

3. Determination of carotenoids in milk

Two hundred µL of milk will be diluted with 1200 µL 2-propanol containing the internal standard Astaxanthin and BHT as an antioxidant. After thorough mixing (15 min) and centrifugation (10 min, 4000 g at 10 °C), an aliquot of 25 µL will be injected from the supernatant into the HPLC system. HPLC will be performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP1100 diode array detector set to 453 nm. Carotenoids will be separated on a 2.1 mm x 150 mm reversed phase C-30 column. The column temperature will be 50 °C. A two-point calibration curve is made from analysis of milk calibrators with known carotenoid concentration. Recovery is higher than 95 %, the method is linear from 0.03 to 3 µM at least and the limit of detection is 0.04 µM. RSD is 2.0-11.3 %.

Analysis of fatty acids in milk

Milk samples for fatty acid analyses are 3-mL aliquots stored at -20 °C until the end of the experiment before lyophilization (ThermovacTM-20, Froilabo, Ozoir-la-Ferriere, France). Fatty acids in lyophilized milk will be directly methylated with 1 mL of 2 N methanolic NaOCH₃ at room temperature for 20 min, followed by 1 mL of 14 % boron trifluoride in methanol at room temperature for 20 min (Christie *et al.*, 2001). Fatty acid

methyl esters will be recovered in 1 mL of hexane. Tricosanoate (Sigma, Saint-Quentin Fallavier, France) will be used as the internal standard. Samples will be injected by auto-sampler into a Trace-GC 2000 series gas chromatograph equipped with a flame ionisation detector (Thermo Finnigan, Les Ulis, France). Methyl esters from all the samples will be separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil 88, Chrompack, Middelburg, The Netherlands). The injector temperature will be maintained at 250 °C and the detector temperature at 255 °C. The initial oven temperature will be held at 70 °C for 1 min, increased by 5 °C/min to 100 °C (held for 2 min), and then increased by 10 °C/min to 175 °C (held for 40 min), and 5 °C/min to a final temperature of 225 °C (held for 15 min). The carrier gas will be hydrogen. A reference standard butter (CRM 164, Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to estimate correction factors for short-chain FA (C4:0 to C10:0).

Satisfactory separations of cis- and trans-18:1, nonconjugated 18:2, and CLA isomers were obtained with a single chromatographic run (see Figure 1 in Loor *et al.*, 2004). Identification of trans-C18:1, non-conjugated C18:2, and CLA isomers was as described in Loor *et al.* (2005).

Analysis of the volatile organic compounds of cow milk fat

Collection and preparation of the milk samples. The milk samples dedicated to the volatile organic compounds analysis are collected in specific 200 mL glass flasks that permit to avoid the contaminations by the flasks. The analyses will be achieved on milk fat extracted from the milk by centrifugation. The milk fat samples are then frozen at -20 °C until the analyses. The 375 milk samples will first be analysed by SPME-gas chromatography-quadrupole mass spectrometry (SPME-CG-MS). This first step will permit to select the more interesting samples that will also be analysed by SPME-Comprehensive two-dimensional gas chromatography-time of flight mass spectrometry analysis (2DGC–TOF-MS).

1. SPME-gas chromatography-quadrupole mass spectrometry analysis (SPME-GC-MS) and construction of corresponding virtual SPME-MS fingerprints

Addition of standards to cow milk fat. As described by Deport *et al.* (2006), six standards will be used to correct the instrumental drifts of the SPME-GC-MS system: 2-methyl-pentane (S1; purity 99.5 %), 1-bromo-butane (S2; purity 99.7 %), fluoro-benzene (S3; purity 99.7 %), bromo-benzene (S4; purity 99.5 %), 1-fluoro-naphtalene (S5; purity 99.0 %) and 1-phenyl-nonane (S6; purity 99.8 %) (Sigma-Aldrich Chimie, St.-Quentin-Fallavier, France). The retention index of these standards is distributed evenly over the sample GC chromatograms. A mixture of the six standards will be prepared in order to obtain an appropriate final concentration of standard co-analysed with each sample.

Analysis parameters. The cow milk fats kept at -20 °C will be installed on a Peltier tray cooler (Gerstel, Mülheim an der Ruhr, Germany) set at 6 °C. The extraction of volatile compounds will be carried out using a model MPS2 multi-purpose sampler (GERSTEL, Baltimore, MD, USA) which manage the following steps: pre-heating of the sample during 10 min at 110 °C in the stirrer (500 rpm), trapping of the volatile compounds of the headspace during 30 min at 110 °C with a 75 µm carboxen-polydimethylsiloxane SPME fiber for Merlin Microseal (Supelco, Bellefonte, PA, USA) and thermic desorption of the trapped volatile compounds by introduction of the fiber in the GC injector. The compounds condensed at the head of the column will be analyzed by a model 6890 GC (Hewlett-Packard, PA, USA) after heating the interface for 2 min at 280 °C and automatic splitless injection onto a 60 m × 0.32 mm i.d., 1 µm, SPB5 capillary column (Sigma Aldrich, Saint Louis, MO, USA). The oven temperature will be successively held at 40 °C for 5 min, increased to 190 °C at a gradient of 3 °C min⁻¹, and further increased to 230 °C for 2 min according to a gradient of 10 °C min⁻¹. The GC column will be connected to a model 5973A mass spectrometer (Hewlett-Packard). The temperature of the column in the transfer section between the GC oven and MS source will be 280 °C. The temperature will be fixed at 180 °C in the MS source and at 150 °C in the MS quadrupole. The electron impact energy will be set at 70 eV and data will be collected in the range of m/z 33 to 230 at a scan range of 1.68 scan s⁻¹.

Fingerprint data treatment. SPME-MS fingerprints of the volatile fraction will be constructed according to previous studies (Vasta *et al.*, 2007 ; Ratel *et al.*, 2008) : the mass spectra which will be acquired every 150ms of the SPME-GC-MS chromatogram and summed, resulting in a virtual SPME-MS fingerprint characterized by the abundance of 198 summed mass fragments ranging from m/z 33 to 230.

Treatment of the data related to GC-MS analyses of milk volatile compounds. The data will be corrected from instrumental drifts according to the Comprehensive Combinatory Standard

Correction (Engel and Ratel, 2007). Pre-processed data will then be treated according to Engel *et al.* (2008) and Lehallier *et al.* (2008).

2. SPME-Comprehensive two-dimensional gas chromatography-time of flight mass spectrometry analysis (2DGC-ToF-MS).

Analysis parameters. The extraction of volatile compounds of the cow milk fatty phases will be carried out according to the same procedure than the SPME-GC-MS analysis described above. The separation, the detection and the identification steps of the volatile compounds will be performed using a LECO Pegasus 4D GC×GC-TOF-MS instrument (LECO, St. Joseph, MI, USA). This system consists of an Agilent 6890N GC, in which two distinct columns separated by a dual stage jet cryogenic modulator were installed, and a time-of-flight MS LECO Pegasus III. The first column is a 30 m × 0.25 mm × 0.25 μm non-polar capillary column (DB-5, J&W, Agilent, Santa Clara, USA). The second column installed in a secondary separate oven is a 2 m × 0.1 mm × 0.1 μm polar column (DB-17, J&W, Agilent). The flow rate is 1 mL min⁻¹. The primary oven temperature is programmed from 40 °C (5 min) to 230 °C (10 min) at 3 °C min⁻¹. The temperature of the secondary oven is adjusted 5 °C higher than the primary oven temperature: 45 °C for 2 min then increased to 235 °C for 10 min, according to a gradient of 3 °C min⁻¹. The modulation time is 7 s, the hot pulse time set to 0.8 s and the modulation temperature offset held to 30 °C. The MS transfer line temperature will be 250 °C and the MS source temperature will be 150 °C. The electron impact energy will be set at 70 eV and data will be collected in the range of m/z 33 to 230 at a scan range of 100 scan s⁻¹ with a voltage of -1650V. Total ion chromatograms (TIC) will be processed using the automated data processing software ChromaTOF (LECO) at signal-to-noise threshold 100. Contour plots will be used to evaluate the general quality of the separation and for manual peak identification. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 - Mainlib and Replib) will be used.

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