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Evolution of free fatty acid profile during ripening in cheeses manufactured from bovine, ovine and caprine milks with extracts of *Cynara cardunculus* as coagulant

Abstract Changes in the concentrations of free fatty acids (FFA) in bovine, ovine and caprine milk cheeses manufactured with a plant rennet (flowers of Cynara cardunculus) were studied throughout a 68-day ripening period. The long-chain saturated (C16:0 and C18:0) and unsaturated (C18:1, C18:2, and C18:3) FFA were the most abundant at all stages of ripening. The overall concentration of FFA in fresh cheese was 3598, 3538 and 3868 mg/kg cheese for bovine, ovine and caprine milk cheeses, respectively; these values increased to 5047, 6517 and 5257 mg/kg cheese, respectively, by 68 days, of which 1171, 1734 and 1791 mg/kg cheese, respectively, were accounted for by C4:0–C12:0. The FFA that showed the highest fractional increase by 68 days of ripening in bovine milk cheese were C4:0, C6:0, C8:0, C12:0, C18:1 and C18:2; in ovine milk cheese they were C4:0, C6:0, C8:0, C10:0, C14:0, and C18:1; and in caprine milk cheese they were C4:0, C8:0, C10:0, C14:0 and C18:1.

Key words Lipolysis · Cow's milk cheese · Ewe's milk cheese · Goat's milk cheese · Thistle

Introduction

Evaluation of the extent of lipolysis is achieved via measurement of the concentrations of free fatty acids, i. e. the products released by such lipase-catalysed reactions when their natural substrates are considered [1]. Free fatty acids (FFA) contribute to the flavour characteristics of many types of cheese, either directly or indirectly, since they are precursors for the formation of several aroma components, e. g. methyl ketones, lactones and aliphatic and aromatic esters [2]. Lipases that contribute to lipolysis in cheese [3] originate from three major sources: milk, rennet and adventitious and/or deliberately added microorganisms [4]. Several microorganisms which are a part of the native microflora of a raw milk cheese have been reported to display lipolytic activity [5].

Although bovine, ovine and caprine milks contain the same basic constituents, ovine milk has considerably higher levels of protein and fat [6, 7], and this fact is of great importance for the manufacture of cheese. Bovine, ovine and caprine milk fats contain high proportions of short- and medium-chain fatty acid residues such as butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0) and lauric (C12:0) acids, but ovine and caprine milk fats have approximately twice the content of these fatty acids when compared with their bovine counterpart [6, 8]; in ovine milk fat the proportion of polyunsaturated fatty acids is lower [9] and the absence of b-carotene [10] is responsible its whiter appearance. The profiles of FFA in bovine, ovine and caprine milks have been reported by several researchers [11–14], and all have shown that the highest concentrations of FFA in milk are accounted for by palmitic and oleic acids, intermediate concentrations by myristic and stearic acids, and the lowest concentrations by caproic, caprylic, linoleic and linolenic acids.

Macedo and Malcata [15] have shown that in Serra, a Portuguese cheese manufactured with raw ovine milk coagulated with extracts of flowers of Cynara cardunculus, the most concentrated FFA throughout ripening are, according to chain length and saturation degree, butyric (short-chain), capric acid (medium-chain), palmitic and stearic acids (saturated long-chain), and oleic acid (unsaturated long-chain). The influence of the type of rennet (animal source vs plant source, i. e. flowers of Cynara cardunculus) on the rate of release of the major fatty acid residues in raw ovine milk cheese was evaluated throughout 68 days of ripening [16], and the overall concentration of FFA by the end of that period was 6517 and 7802 mg/kg cheese for the plant rennet and the animal rennet, respectively. The aim of this study was to eventually complement such studies via comparison of the FFA profiles of bovine, ovine and
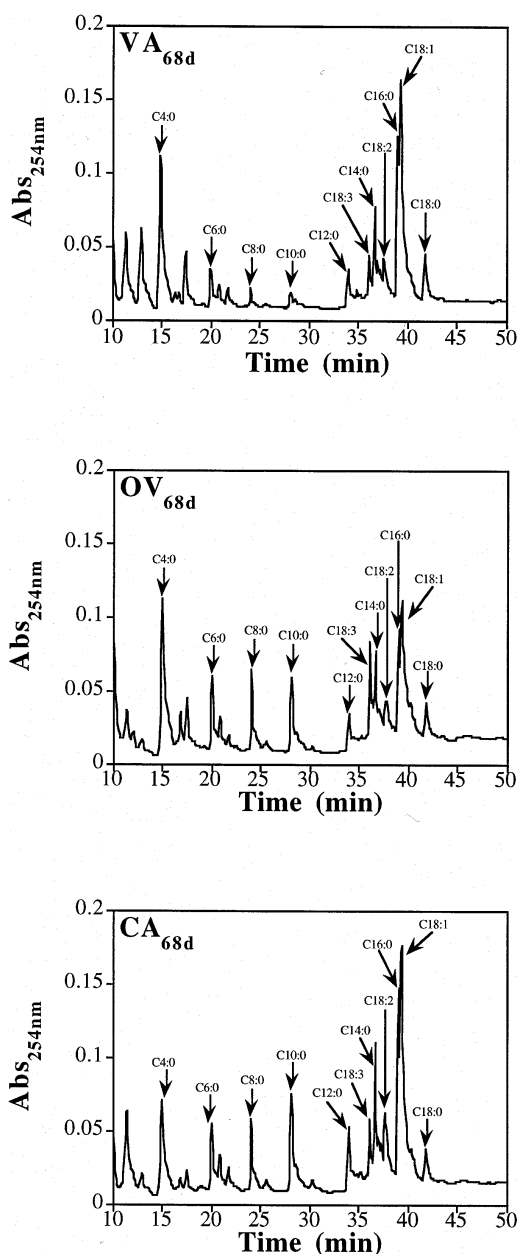
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Fig. 1 HPLC qualitative profile (expressed as absorbance of the eluate measured at 254 nm as a function of elution time in min) of free fatty acids in bovine (VA), ovine (OV) and caprine (CA) milk cheeses manufactured with plant coagulant (Cynara cardunculus) by 68 days of ripening (without C9:0 and C17:0 added as internal standards)

caprine milk cheeses manufactured with extracts of Cynara cardunculus as plant coagulant throughout the ripening period.

Materials and methods

Enzyme extract. Dried flowers of wild thistle (Cynara cardunculus) were obtained at random from local shops in Serra da Estrela region (Portugal). The crude extract was prepared by grinding the stylets of

the flowers for 45 s, suspending in distilled water and stirring for 15 min, and filtering through a fine piece of cloth.

Cheesemaking. Raw bovine, ovine and caprine milk cheeses were manufactured using the enzyme extracts prepared as above as coagulant following an adaptation of the traditional technology [7, 17]. The raw milk (from bovine, ovine or caprine origin) was heated to 28 °C, salted (3 g/l), and left to coagulate for 50 min by addition of 0.16 g of crude stylets/l. The curd was cut, stirred for 30 min, allowed to set to promote whey drainage, placed into cylindrical moulds and lightly pressed by hand. The cheeses were salted with dry salt on both surfaces, and 24 h later were placed in a ripening room kept at 6 °C and 92 % relative humidity. After 14–20 days the cheeses were washed with warm salted water; the cheeses were turned upside down daily for 68 days. In total, 24 cheeses of each type (500 ± 100 g in weight, 100.0 ± 10.0 mm in diameter and 50 ± 10.0 mm height) were thus manufactured and duly ripened.

Cheese sampling. One cheese from each type (i. e. from bovine, ovine or caprine milk) was taken at random at 0, 7, 14, 28, 42, 56 and 68 days of ripening. Then, 5 g taken at random from that cheese was mixed using a vortex in a screw-capped test tube with 2.5 g of anhydrous sodium sulphate (Merck, Darmstadt, Germany) and 10 ml of diethyl ether (Merck) and variable amounts (depending on the ripening time) of internal standard solution, as detailed below. This mixture was stirred in the vortex for 1 min every 1 h for a period of 4 h, and homogenized in a Sonorex RK100 (Bandelin, Berlin, Germany) for 15 min every 1 h.

FFA assaying. The FFA in the experimental samples were analysed by HPLC using a procedure initially developed by Garcia et al. [18] and later modified by Balcão and Malcata [19].

For calibration, stock solutions of 12 FFA standards, i. e. C4:0 (butyric acid), C6:0 (caproic acid), C8:0 (caprylic acid), C10:0 (capric acid), C12:0 (lauric acid), C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (g-linolenic acid) and C20:0 (arachidic acid) (Sigma, St. Louis Mo., USA), were prepared by weighing given amounts of the said fatty acid chromatographic standards and dissolving them in a 1:1 (v/v) methanol/chloroform mixture (Romil, Shepshed, UK), so as to obtain a final (known) concentration of ≈0.2 M for each FFA standard. Aliquots of 100 ml of all stock solutions of fatty acid standards were taken and added to enough volume of 1:1 (v/v) methanol/chloroform so as to obtain a total fatty acid standard volume of 6.0 ml. A volume of 4.5 ml of a solution of internal standards containing 1 g of C9:0 (nonanoic acid) and 2 g of C17:0 (heptadecanoic acid) (Sigma) in a 1:1 (v/v) methanol/chloroform mixture stabilized with 0.05% butylated hydroxyanisole (Sigma) was then added, so as to obtain a final 4:3 ratio of fatty acid standard volume to internal standard volume. This procedure was independently used for other aliquots of the fatty acid standard stock solutions, i. e. 200, 300, 400 and 500 ml. Aliquots (100 ml) of each resulting solution were withdrawn and added to 4.0 ml of a 1 g/l solution of p-bromophenacylbromide (Sigma) in acetonitrile (Romil). To the resulting solution, 80 ml of a 5 g/l solution of 18-crown-6-ether (Merck) in acetonitrile was added, and this procedure was followed by addition of 0.2 g of potassium carbonate (Merck). After thorough mixing, this biphasic mixture was incubated at 75–80 °C for 30 min, allowed to cool to near room temperature, then 40 ml of a 40 g/l solution of formic acid (Romil) in acetonitrile was added, and finally incubated at 75–80 °C for an additional period of 5 min. After refrigeration at ≈4 °C for at least 1 h, samples were cold-filtered through 0.45-nm nylon membrane filters. Aliquots of 20 ml of the filtered samples were then injected into the HPLC system (Beckman Instruments, San Ramon, Calif., USA) which consisted of an autosampler with temperature control for the column (model 502), a C-18 reversed-phase column (25 cm· 4.6 mm· 5 nm) coupled with a pre-column cartridge (4.5 cm· 4.6 mm· 5 nm), a solvent delivery system with two pumps (Programmable Solvent Module 126), a programmable multiwavelength spectrophotometer (Diode Array Detector Module 168), and a software package for system control and data acquisition (GOLD v6.01); separation was effected at 33 °C using a mobile phase of

water, methanol and acetonitrile under a gradient system; the flow rate of eluant was 1 ml/min; and absorbance of the eluate was read at 254 nm.

For assaying of actual cheese samples, 1.5 ml of the diethyl ether cheese extract was taken and mixed with 4 ml of a 1 g/l solution of p-bromophenacylbromide in acetonitrile. After this point, the derivatization procedure was the same as for the calibration.

Results and discussion

The concentrations of free nonanoic (C9:0) and heptadecanoic (C17:0) acids in bovine, caprine and ovine milk cheeses have been reported to be rather low [14] and even negligible [8, 20]. Preliminary analyses confirmed that these acids do not exist in bovine and caprine milk cheeses above the threshold of sensitivity, but that they are present in ovine milk cheeses at trace levels (see Fig. 1). Therefore, use of these compounds as internal standards (as done in the present work) seems essentially acceptable from an analytical point of view.

Changes in the concentration of individual FFA with ripening time for bovine, ovine and caprine milk cheeses are plotted in Fig. 2. The highest concentrations of FFA in fresh cheeses are accounted for by C18:1 (694.6, 465.1 and 510.9 mg/kg cheese for bovine, ovine and caprine milk cheeses, respectively), C18:0 (688.6, 999.0 and 852.0 mg/kg cheese), and C16:0 (820.4, 695.2 and 857.4 mg/kg cheese). Intermediate concentrations are accounted for by C18:2 (319.2, 219.1 and 330.8 mg/kg cheese) and C18:3 (267.9, 445.2 and 344.1 mg/kg cheese). The lowest concentrations are accounted for by C12:0 (160.3, 111.3, and 162.9 mg/kg cheese), C10:0 (145.7, 194.4 and 239.4 mg/kg cheese), C8:0 (115.98, 171.3 and 153.4 mg/kg cheese), C6:0 (80.3, 79.2 and 201.9 mg/kg cheese), and C4:0 (197.3, 157.9 and 142.4 mg/kg cheese); C14:0 is present in bovine and caprine milk cheeses (107 and 73.2 mg/kg cheese) but not in ovine milk cheese at 0 days of ripening, an observation that agrees with results obtained by Sousa and Malcata [16] and Macedo and Malcata [15] pertaining to Serra cheese. The concentrations of all individual FFA increased as ripening progressed. Although C16:0, C18:0 and C18:1 were the most abundant FFA at all stages of ripening for all types of cheese, long-chain fatty acids do not contribute to cheese flavour nearly as much as short-chain fatty acids do [21]. In other varieties of bovine (Mahon), ovine (Manchego and Roncal) and caprine (Majorero) milk cheeses, C18:1, C18:0, C16:0, C14:0 and C4:0 have been reported to be the most abundant FFA [14]; these results are consistent with the FFA profile obtained in our research effort, which is also consistent with the FFA profile of ovine milk cheese manufactured with plant coagulant [16], of Serra cheese [15], of Spanish ovine milk cheeses [12] and of Italian ovine milk cheeses [13].

During the ripening period, the FFA that showed the highest fractional increase in bovine cheese were C4:0, C6:0, C8:0, C12:0, C18:1 and C18:2 (2.24, 1.50, 1.55, 1.64, 1.50 and 1.75, respectively); those in ovine milk cheese were C4:0, C6:0, C8:0, C10:0, C14:0 and
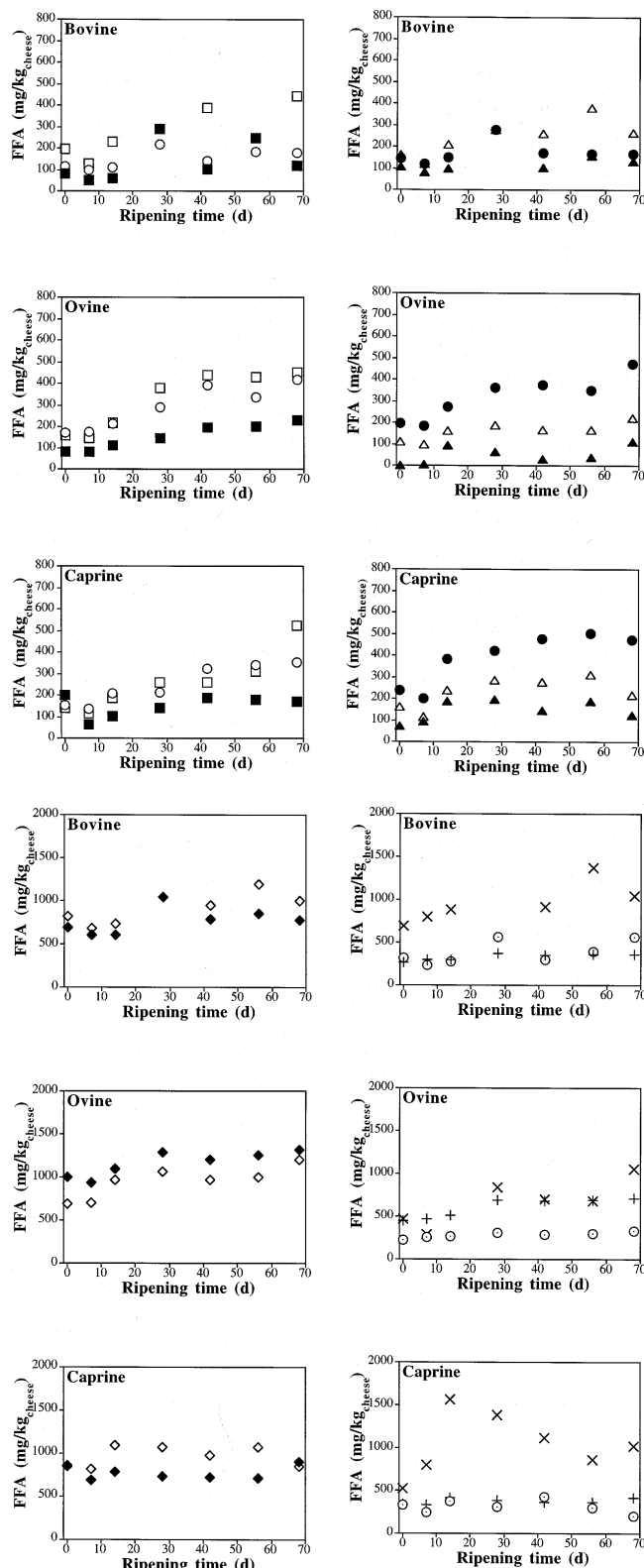

Fig. 2 Changes in concentration of individual free fatty acids (FFA) throughout ripening: short-chain FFA [C4:0 (□), C6:0 (■), and C8:0 (○)], medium-chain saturated FFA [C10:0 (●), C12:0 (△), and C14:0 (▲)], long-chain saturated FFA [C16:0 (◇) and C18:0 (◆)], and long-chain unsaturated FFA [C18:1 (·), C18:2 (○), and C18:3 (+)], in bovine, ovine and caprine milk cheeses manufactured with plant coagulant (Cynara cardunculus)

C18:1 (2.85, 2.90, 2.47, 2.43, 6.89 and 2.26, respectively) whilst those in caprine milk cheese were C4:0, C8:0, C10:0, C14:0 and C18:1 (3.68, 2.29, 1.97, 1.67 and 1.99, respectively). These results agree with the fact that lipases (either from milk, rennet or microbial origin) involved in cheese ripening possess a higher selectivity towards hydrolysis of short- and medium-chain fatty acid residues than towards long-chain ones [14].

The overall FFA concentrations in cheese were 3598, 3538 and 3868 mg/kg cheese on day 0 of ripening for bovine, ovine and caprine milk cheeses, respectively, and 5047, 6517 and 5257 mg/kg cheese by 68 days of ripening; therefore, the overall FFA concentration increased by 1449, 2979 and 1389 mg/kg cheese for bovine, ovine and caprine milk cheeses, respectively. The higher increase in the concentration of FFA in ovine milk cheeses than in bovine or caprine milk cheeses can be partly derived from the fact that ovine milk cheeses contain higher levels of fat (63.6% of total solids, TS) than bovine (41.0% TS) or caprine (45.1% TS) milk cheeses [7]. The FFA ranging from C4:0 to C12:0 accounted for 1171, 1734 and 1791 mg/kg cheese in bovine, ovine and caprine milk cheeses, respectively, of the overall FFA concentrations of 5047, 6517 and 5257 mg/kg cheese obtained by 68 days of ripening; the qualitative and quantitative profiles of such short-chain FFA have been claimed to provide dairy products with their unique organoleptic properties, and so the aforementioned fractional amount can be regarded as a useful index in characterizing cheeses throughout the ripening period irrespective of the origin of the milk from which they are made.

Despite the differences between the various experimental cheeses, lipolysis can in general be asserted as being relatively low in bovine, ovine and caprine milk cheeses by 68 days of ripening. Similar results were obtained for ovine cheese [16], Serra cheese [15] and Serena cheese [22], all of which were manufactured with ovine milk and extracts of Cynara spp. as coagulant. Therefore, the lipolytic activity contained in such plant rennet can be considered as low, as would be expected from the best animal rennets [16].

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