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A survey of the main technology, biochemical and microbiological features influencing the concentration of biogenic amines of twenty Apulian and Sicilian (Southern Italy) cheeses

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ABSTRACT

Twenty Apulian and Sicilian cheeses were analysed for their concentrations of eight biogenic amines (BAs), free amino acids, pH, water activity, and subjected to microbiological characterisation. In addition, lactic acid bacteria isolated from cheeses were assayed for their capacity to generate BAs. Principal component analysis was performed to find the effect of different parameters on the distribution of the cheeses. Although short-ripened (≤30 d) cheeses did not show significant BA concentrations, the only BA showing high positive correlation with time of ripening was histamine. Concentration of histidine and, especially, percentage of histidine-decarboxylase bacteria presumably affected histamine concentration. High pH values were negatively correlated to the concentration of tyramine, putrescine, and cadaverine. Fifty percent of the cheeses contained at least one BA at potentially toxic concentrations. Unambiguous and ever-valid relations among parameters and BAs are difficult to determine, because BAs are the result of combined and varied factors.

1. Introduction

Biogenic amines (BAs) are low-molecular nitrogenous organic bases that are formed in foodstuffs by microbial decarboxylation of the precursor amino acids. Tyrosine, histidine, lysine, tryptophan, and phenylalanine are, respectively, precursor amino acids for tyramine, histamine, cadaverine, tryptamine, and 2-phenylethylamine. Ornithine and arginine may be the precursors for putrescine, spermidine and spermine. Although small amounts of BAs are biosynthesised in plant and animal cells, having different biological activities (Pinho et al., 2004), these compounds are potentially toxic to human health. The effects on nervous and vascular systems are particularly severe in sensitive people or when the amine oxidases, naturally involved in the detoxification, are inhibited (Shalaby, 1996; Silla Santos, 1996). The toxicity limit of BAs in foods is estimated to be 100 mg kg^{-1}, even though it is stated that the safe sum of histamine, tyramine, putrescine and cadaverine should not exceed 900 mg kg^{-1} (Shalaby, 1996; Valsamaki, Michaelidou, & Polychroniadou, 2000).

Cheeses are among the foods most commonly associated with the presence of BAs (Innocente & D'Agostin, 2002; Moret, Bortolomeazzi, Feruglio, & Lerker, 1992; Stratton, Hutkins, & Taylor, 1991). Indeed, the main biochemical process that takes place during cheese ripening, proteolysis, leads to the accumulation of free amino acids (FAAs), some of which are precursors of BAs. The BA concentration of many typical and/or traditional Italian cheeses was analysed (Innocente, Biasutti, Padovese, & Moret, 2007; Ladero, Fernández, & Álvarez, 2009; Martuscelli et al., 2005; Schirone et al., 2013; Spizzirri et al., 2013). Overall, the concentration and type of BAs in cheeses is extremely variable, depending on: (i) type of milk (cows'/sheep's/goats' milk); (ii) thermal treatment of cheese milk; (iii) section of the cheese (edge/core); (iv) ripening conditions; (v) post-ripening processing; (vi) type of packaging; (vii) storage time and temperature; and (viii) microbiota responsible for cheese-making (Loizzo et al., 2013). Generally, the concentration of BAs was lower in short-ripened than in long-ripened cheeses (Bunková et al., 2010; Fernández, Linares, Del Rio, Ladero, & Alvarez, 2007), where the level of proteolysis and catabolism of

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FAAs increases. Cheeses possess very rich, diverse and complex microbiota, mainly deriving from primary starter lactic acid bacteria and adventitious non-starter lactic acid bacteria (NSLAB; Beresford & Williams, 2004). High cell densities of NSLAB, possessing amino acid decarboxylating enzymes (e.g., tyrosine decarboxylase), were positively correlated with high content of BAs in cheeses (Bunková et al., 2010; Fernández et al., 2007; Komprda et al., 2008; Ladero et al., 2009; Martuscelli et al., 2005). Nevertheless, the concentration of ethylamine, tryptamine, 2-phenylethylamine, and cystamine during ripening of Terrincho cheese, reached the maximum at 30 days, but subsequently decreased, meaning that such compounds are also degraded or transformed (Pinho et al., 2004).

Although, except for histamine in fish products, there is no consensus on the maximum permitted concentration of BAs in foods, everybody acknowledges that reducing the concentration of such potentially toxic compounds in foods is extremely important. The formation of BAs may be limited through the use of either amine-negative (not able to decarboxylate FAAs into BAs) or amine oxidising starter cultures (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000, 2001; Latorre-Moratalla et al., 2007; Mah, Kim, & Hwang, 2009; Spicka, Kalac, Bover-Cid, & Krizek, 2002; Stratton et al., 1991).

To the best of our knowledge, no studies were focused on the concentration of all of the possible BAs in a high number of traditional cheeses, and looking at this food safety feature as possibly affected by technology parameters, concentration of precursor FAAs and microbiological characteristics. Therefore, the aim of this study was to correlate the content of BAs of twenty traditional Apulian or Sicilian (Southern Italy) cheeses with several technological and microbiological features such as time of ripening, pH, concentration of precursor FAAs, and occurrence of decarboxylase-positive lactic acid bacteria.

2. Materials and methods

2.1. Cheeses

Nine Apulian (Cacio, Caciocavallo Podolico Dauno, Caciocavallo Silano Protected Designation of Origin (PDO), Cacioricotta, Canestrato Pugliese PDO, Caprino di Biccari, Caprino di Castel Fiorentino, Pecorino Foggiano, and Vaccino), and eleven Sicilian (Caciocavallo Palermitano, Ragusano PDO, Caprino Girgentano, Fior di Capra, Fiore Sicano, Maiorchino, Pecorino Siciliano PDO, Piacentinu Ennese, Provola dei Nebrodi, Tuma Persa, and Vastedda della valle del Belice PDO) traditional cheeses were evaluated in this study. Table 1 summarises the main parameters used during manufacturing and ripening, as well as the approximate moisture level of each cheese. Three batches of each cheese were collected at local dairy farms and kept at 4 °C during transfer (1–3 h) to laboratory. Each cheese was analysed for BAs, pH, water activity (a_w), FAAs, and microbiological features.

2.2. Determination of biogenic amines in cheeses

BAs were extracted from cheese, derivatised and quantified, according to the method described by Innocente et al. (2007), with modifications. In detail, 10 g of cheese were weighed in a 50 mL polypropylene tube and 20 mL of 0.1 mol L^{-1} HCl added, containing 1,7-diaminoheptane (0.01 mg mL^{-1}) as the internal standard. The suspension was homogenised for 2 min in a BagMixer 400P (Interscience, St. Nom, France) blender. The homogenate was centrifuged at 15,557 × *g*, at 4 °C, for 30 min and the supernatant (first acid extract) was transferred into a clean 50 mL polypropylene tube. The pellet was added with 20 mL of 0.1 mol L^{-1} HCl, containing the internal standard, homogenised and centrifuged as described above. The supernatant (second acid extract) was recovered and mixed with the first acid extract. The two extracts were diluted to 50 mL with 0.1 mol L^{-1} HCl.

Extracted BAs were derivatised by mixing 1 mL of extract, 0.5 mL of saturated $NaHCO_3$ solution and 1 mL of dansyl chloride (DCl) reagent (dissolved in acetone at 10 g L^{-1}) in a 15 mL polypropylene tube, protected from light. The reaction mixture was then left for 60 min at 40 °C and vortexed at 15 min intervals. Excess of DCl was removed by addition of 0.3 mL of ammonia solution (300 g L^{-1}), vortexing for 1 min and leaving to react in the dark for 15 min at room temperature. The sample was extracted twice (duration of each extraction: 5 min) with 1 mL aliquot of diethyl ether. The combined extracts were collected in 2 mL polypropylene tubes and dried (110 min, room temperature) in a vacuum centrifuge (SpeedVac Concentrator SPD121P, Thermo Fisher Scientific, Marietta, OH, USA). Finally, the residue was re-dissolved in 1 mL of acetonitrile for injection.

BAs were separated using an Äkta Purifier 10 (GE Healthcare Bio-Sciences, Uppsala, Sweden), equipped with a 20 μL loop, a reverse phase C_{18} column (Kromasil 100 A, 5 μm, 4.6 × 250 mm, StepBio, Bologna, Italy) thermostated at 30 °C, with a guard cartridge Kromasil 100-5C18, and a UV detector at 254 nm. BAs were eluted at 0.8 mL min^{-1} with acetonitrile (A) and water (B), using the following gradient: 65% A (1 min), 65–80% A (9 min), 80–90% A (2 min), 90–100% A (4 min), 100% A (7 min) (Moret, Smela, Populin, & Conte, 2005). BAs were quantified using calibration curves built up after having analysed standard solutions, containing the following amines dissolved in 0.1 M HCl at concentrations of 2, 5, 10, and 20 μg mL^{-1}: cadaverine, histamine, 2-phenylethylamine, putrescine, spermidine, spermine, tryptamine and tyramine. The following formula was used:

$$\text{Concentration of BAs}\left(\text{mg kg}^{-1}\text{of cheese}\right) = [(\text{BA peak area}/\text{internal standard peak area}) - q]/m$$

where q and m are the parameters of the calibration curve for that amine. Prior to HPLC analysis, the standard solutions were derivatised under the same conditions as the acid extracts, except for the concentration of DCl (5 g L^{-1}).

2.3. Determination of pH, water activity, and free amino acids in cheeses

The value of pH was determined by direct insertion of a Foodtrode (Hamilton, Bonaduz, Switzerland) electrode. Water activity (a_w) was determined using the Dew Point Water Activity Meter AquaLab (Mod. 4TE, Decagon Devices, Inc., Pullman, WA, USA), according to the manufacturer's instructions.

Concentration of individual FAAs in cheese was determined as described by Siragusa et al. (2007), with few modifications. In detail, 30 g of cheese was grated and homogenised (5 min treatment) with 90 mL of 50 mmol L^{-1} phosphate buffer, pH 7.0, in a blender. The suspension was kept at 40 °C for 1 h under gentle stirring (150 rpm) and centrifuged at 1157 × *g* for 30 min at 4 °C. The supernatant was collected and centrifuged (1157 × *g*, 10 min, 4 °C). One millilitre of the supernatant from the second centrifugation was added with 50 mg of cold sulphosalycillic acid (final concentration: 50 mg mL^{-1}) and incubated for 1 h at 4 °C to precipitate proteins and most of peptides. After centrifugation (23,000 × *g*, 15 min), the extract, containing just FAAs, was filtered (Mini-Uni PrepTM, pore size 0.2 μm, GE Healthcare Life Science), diluted (if needed) with sodium citrate loading buffer, and injected (20 μL) into a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK), equipped with a sodium cation-exchange column (20 by 0.46 cm [inner diameter]). Amino acids were post-column