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Aflatoxin M_1 in the intermediate dairy products from Manchego cheese production: distribution and stability

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Summary

Aflatoxin M_1 (AFM_1) distribution in curd, whey, Manchego cheese, the traditional Spanish whey cheese Requesón and Requesón whey, and its stability during two different cold treatments, have been studied. Raw ewe's milk was artificially contaminated with AFM_1 in a final concentration of 50 and 100 ng kg^{-1}, and was used to produce Manchego cheese. AFM_1 determinations were carried out by HPLC with fluorimetric detection after immunoaffinity clean-up. The mean AFM_1 concentrations in the produced curd and Manchego cheese were approximately 2- and 3-fold higher than the initial milk they were made from, and the levels of this toxin remaining in whey were 42.3 % and 51.3 % of the initial concentrations. In the Requesón samples, the mean AFM_1 values were 1.7 times higher than those in the corresponding whey, while 33.7 % and 44.4 % of the AFM_1 concentration detected in milk also appeared in the Requesón whey. Short refrigeration and freezing periods did not affect the toxin levels in either curd or Requesón samples. When ewe's milk destined for Manchego cheese-making is AFM_1-contaminated at the EU limit level (50 ng kg^{-1}) or double, a concentration of this toxin will appear in the manufactured products, but values will be considerably below the toxic doses (Tolerable Daily Intake = 2 ng kg^{-1} body weight per day), which poses a human health problem.

Key words: aflatoxin M_1, ewe's milk, curd, whey, cheese

Introduction

Aflatoxins (AFs) are highly toxic secondary metabolites produced by several *Aspergillus*, *Penicillium* and *Rhizopus* species in crops and plant products, of which aflatoxin B_1 (AFB_1) is the most representative (Goldblatt, 1969). If AFB_1 is present in the feed of lactating animals, they will excrete aflatoxin M_1 (AFM_1) into their milk (Allcroft and Carnaghan, 1963; Cupid et al., 2004; Battacone et al., 2005), a toxin classified by the International Agency for Research on Cancer as class 2B, possible human carcinogen (IARC, 2002). Given AFM_1 implications to consumers' health in relation to milk, the Codex Alimentarius set a maximum recommended level of 500 ng kg^{-1} (Codex Alimentarius, 2001), although a stricter limit (50 ng kg^{-1}) for raw milk, milk for the production of milk-based products and heat-treated milk was established in the European Union (Commission Regulation, 2003). Regarding cheese, only a few countries (The Netherlands, Switzerland, Austria, Turkey and Italy) have specified limits within the range of 200-450 ng kg^{-1}. In Spain, no regulation to control the presence of AFM_1 in dairy products exists, even though it has a deep-rooted cheese-making tradition. Moreover, few studies (López et al., 1996) have been conducted on products with a protected designation of origin (PDO), such as Manchego cheese.

When cheese-making is carried out using AFM_1-contaminated milk, this toxin is likely to have become enriched in the final curd compared to that found in milk. This could be explained by both the capacity of AFM_1 to somehow bind caseins

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(Applebaum et al., 1982; Battacone et al., 2005) and increased dry matter content (Deveci, 2007). The affinity of AFM_1 has been tested not only with these proteins, but also with other different ones present in whey as a larger amount of this toxin has been demonstrated to be present in the retentive where the protein-rich fraction appears (Mendoça and Venâncio, 2005). Therefore, it is necessary to note whether AFM_1 is present in final products like cheese because its concentration in them has been reported to be around 2.1-4.5 times higher than in the original milk used, depending on the cheese type (Van Egmond, 1983; Wiseman and Marth, 1983; Blanco et al., 1988; Deveci, 2007; Manetta et al., 2009). Nevertheless in different products like yoghurt, the level of AFM_1 is stable and not influenced by yoghurt production (Blanco et al., 1993). In line with this, AFM_1 may also be present in other dairy products obtained from whey, and knowledge of AFM_1 concentration in Requesón is appropriate to ensure food safety.

A partition of AFM_1 occurs between whey and curd, with 40-60 % of the total AFM_1 amount registered in whey if compared to that present in milk (Govaris et al., 2001; Oruc et al., 2006; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009). However, this range has also been found to be higher, between 70 % and 74 % (Battacone et al., 2005), or lower, between 17 % and 27 % (López et al., 2001). This wide range of AFM_1 distribution after cheese-making could be due to different factors; e.g., the extraction technique used before the HPLC analysis (Battacone et al., 2005; Kamkar et al., 2008) which is associated with sample size or the extraction solvent. The characteristics of the manufacturing process, depending on the cheese type, could also influence AFM_1 distribution, as demonstrated in both hard (Brackett and Marth, 1982; Blanco et al., 1988; Manetta et al., 2009) and soft cheeses (Wiseman and Marth, 1983; Govaris et al., 2001; Oruc et al., 2006). In addition, the milk contamination type should also be considered because when milk is naturally contaminated (Virdis et al., 2008), AFM_1 values are usually lower than in artificially contaminated milk (Deveci, 2007).

The present work aimed to investigate AFM_1 distribution in the intermediate dairy products derived from Manchego cheese production, such as curd, whey, Requesón and Requesón whey, which are produced with artificially contaminated ewe's milk; and at the same time to assess the effect of cold treatments on AFM_1 content in curd and Requesón prior to their analysis.

Materials and methods

AFM_1 contamination of milk and cheese-making

Two 10-litre vats of Manchego raw ewe's milk were prepared in duplicate to manufacture Manchego cheese. Milk was firstly analysed without detecting AFM_1. Spiking was performed by addition of AFM_1 standard (R-Biopharm, Glasgow, Scotland (UK), 1024 ng/mL in acetonitrile) to the milk in the vats to obtain concentrations of 50 ng L^{-1} and 100 ng L^{-1} (vat 50 and vat 100), which was than stirred at room temperature (22 °C) for 30 minutes (Oruc et al., 2006) prior to the cheese-making procedure.

In this work, Manchego cheese production followed the protocol of Pavia et al. (1999). This is a ripened and pressed extra-fat cheese with a minimum of 50 % of fat, as described by its Designation of Origin. The cheeses were made with moulds to about 1 kg of curd and put into a 20 % brine solution for 8 hours. Afterwards, they were left to ripen in a maturation chamber for 2 months at 11±1 °C with a relative humidity of 85±2 %.

To produce the typical Spanish whey cheese known as Requesón, the obtained whey was heated with slight yet constant stirring at 90 °C. Having reached this temperature, stirring was interrupted until a temperature of 90 °C was achieved to favour protein coagulation; the proteins floating on the surface were collected with a slotted spoon. The resulting product was transferred to perforated plastic moulds (6 x 6 x 7 cm) weighing approximately 100 g each. Then the Requesón whey was also collected, which was obtained after making this whey cheese. All the products obtained from this cheese-making procedure were analysed for AFM_1 determination.

Refrigeration and freezing

After the cheese-making procedure, 6 curd, 3 Requesón and 1 cheese aliquot(s) were collected from each vat. Two curd and 2 Requesón aliquots were placed into hermetic containers for 2 days at 6±2 °C for the refrigeration experiment, and 3 curd aliquots were frozen for 2, 7 and 14 days, respec-

tively, at -25 °C for the freezing experiment. All the samples were analysed after the preservation times to verify how AFM_1 concentration evolved and to compare it with that obtained on the day that cheese-making took place (day 0).

Composition analysis

The chemical composition analysis (fat, protein, lactose and total solids) of milk was performed at the LICOVAL Laboratory (Valencia, Spain) with a CombiFoss 5000 machine (FossElectric, Hillerød, Denmark), which combines the Milkoscan 4000 (infrared analysis), previously calibrated for ewe's milk, with the Fossomatic 5000 (flow cytometry somatic cell counter), calibrated using certified standards.

The composition (dry extract, fat, moisture and salt) of the curd, cheese and Requesón samples was determined using a Foodscan (FossElectric, Hillerød, Denmark) at the Castilla-La Mancha University's Food Quality laboratory.

AFM_1 determination by HPLC

For the clean-up procedure before the HPLC analysis, immunoaffinity columns (AFLAPREP® M, R-Biopharm Rhône Ltd, Glasgow, Scotland, UK) were used following the manufacturer's instructions. Next, 50 mL of all the milk and whey samples were skimmed by centrifugation, filtered and warmed before use. A brine sample was also taken to be analysed. The curd, Requesón and cheese samples were extracted using the method reported by Battacone et al. (2005). Grated samples (20 g) were extracted with 75 mL of chloroform, 1 mL of NaCl saturated solution and 5 g of Celite 545 by agitation for 45 minutes. After filtration, a rotary evaporator was used at 35-40 °C to evaporate the filtrate. The obtained residue was mixed with 50 mL of hexane, 1 mL of methanol and 30 mL of distilled water. After three washing steps with 10 mL of distilled water, the oily phase was removed, and the aqueous phase was recovered and cleaned up in the aforementioned column.

The AFM_1 content in the samples was determined at the Animal Science and Technology Institute (Polytechnic University of Valencia) following the ISO (2007). Samples were analysed in a Waters HPLC system (Milford, MA, USA) equipped with two 515 pumps, a 717 autosampler and a 745 fluorescence detector. The volume injected into the HPLC system was 100 μL and each extract was injected in duplicate. Separation was performed in a Waters (Milford, MA, USA) Nova-Pak C-18 column (150 mm x 4.6 mm x 5 μm) in 7 minutes at room temperature with a retention time of 4.5 minutes for AFM_1. The mobile phase consisted in acetonitrile-water (25:75, v/v) at a flow rate of 1.0 mL min^{-1}. The excitation and emission wavelengths for the fluorescence detector were 365 and 435 nm, respectively. Standard AFM_1 (R-Biopharm, Glasgow, Scotland, 1024 ng mL^{-1} of AFM_1 in acetonitrile, w/v) was diluted in water-acetonitrile (90:10, v/v) to give a standard stock solution (102.4 ng mL^{-1}). A series of calibration solutions were prepared by diluting standard stock solution in water-acetonitrile (90:10, v/v) to give a daily calibration curve (40, 80, 160, 320, 640, 1280 and 2560 ng L^{-1} of AFM_1), with a detection limit of 10 ng L^{-1}. The peak areas against the injected quantity of AFM_1 were employed for plotting the calibration curve. The data from the calibration curve and the formula included in the ISO (2007) were used to calculate the final AFM_1 concentration in the samples.

Statistical analysis

All the data were treated statistically using SPSS Statistics 17.0 for Windows (SPSS Inc., Chicago, IL, USA). To determine the effect of cold treatments, a General Linear Model was used (GLM) with the equation: $Y_{ij} = \mu + R_i + F_j + \varepsilon_{ij}$; where Y_{ij} is the AFM_1 level, μ is the general mean, R_i is the refrigeration effect, F_j is the freezing effect, and ε_{ij} is the residual error. The mean and standard deviation (SD) were used to express the results of the composition and AFM_1 levels.

Results and discussion

Information about curd, Requesón and cheese compositions is provided in Table 1. All the values for the 2-month ripened cheeses fall in acceptable ranges for Manchego cheese (González et al., 2000), a full-fat ewe's cheese with more than 70 % of fat over dry matter. No data have been published about curd composition because it is an intermediate product from the Manchego cheese-making process, although in the case of Requesón, composition values are similar to those of Licón et al. (2010).

Table 1. Mean values and standard deviations for the composition analyses of the curd, Requesón and cheese samples

Sample	Yield (kg)		Fat[1] (%)		Moisture (%)		Salt (%)	
	50[a]	100[b]	50[a]	100[b]	50[a]	100[b]	50[a]	100[b]
Curd	2.9±1.2	3.1±2.2	54.6±0.0	53.7±0.0	50.5±2.3	51.2±2.9	1.0±0.1	1.2±0.0
Requesón	0.6±2.1	0.5±3.0	42.1±0.1	40.1±0.0	70.3±3.2	71.1±0.1	1.0±0.1	1.1±0.1
Cheese[c]	2.7[c]±1.6	2.7[c]±2.4	55.7±0.0	53.6±0.0	21.1±5.5	22.1±3.7	2.8±0.9	2.8±0.5

[1]Expressed as fat divided by dry matter
[a]AFM_1 contamination of milk at 50 ng/kg
[b]AFM_1 contamination of milk at 100 ng/kg
[c]Manchego cheeses weighing about 1 kg which ripened for 2 months. Values expressed as an estimation of the total amount of curd obtained

The milk used to make cheese presented a mean composition of 7.2 % fat, 5.6 % protein, 5.0 % lactose and 12.8 % cheese extract. After the spiking procedure, the AFM_1 contamination level found in the milk used for the cheese-making process (Table 2) was 47.8 ng kg^{-1} and 80.3 ng kg^{-1} for vat 50 and vat 100, respectively. These values assume the total AFM_1 amounts of 497.1 ng and 811.1 ng per 10 L of milk, which indicate a minimum AFM_1 recovery in the HPLC analysis of over 80 %, a value falling in the acceptable range as suggested by the ISO (2007) for this method.

The curd samples obtained presented an AFM_1 concentration of 108.0 ng kg^{-1} (vat 50) and 161.7 ng kg^{-1} (vat 100), values which represent contamination levels of 2.7- and 2-fold higher than the milk they were made from. After the ripening process, these values in cheese were 2.9- and 2.7-fold higher, which AFM_1 values were 139.0 ng kg^{-1} and 221.3 ng kg^{-1} for vat 50 and vat 100, respectively. These results are comparable with the mean 2.5- to 2.8-fold increases reported by different authors (Wiseman and Marth, 1983; Deveci, 2007; Kamkar et al., 2008) for milk contaminated with 50 ng kg^{-1} to 2800 ng kg^{-1}. This situation suggests that the degree of AFM_1 contamination in milk does not seem to influence the final mycotoxin concentration.

Several studies have been carried out using milk naturally contaminated with AFM_1 to produce cheeses with very distinct characteristics, like Queso Blanco and Bakers' (Wiseman and Marth, 1983), Cheddar (Brackett and Marth, 1982) or Grana Padano (Manetta et al., 2009), with a wider concentration range and increases of 2.5- to 4.5-fold. When considering the manufacturing procedure, some differences can be found in the cheeses made with and without rennet, with a 3- and 4.2-fold increase, respectively, than those found in the milk employed (Wiseman and Marth, 1983). The results obtained for the cheeses produced with rennet are comparable to those in the present work, although the manufacturing process and types of milk differ. A more recent study (Manetta et al., 2009) was conducted on a typical hard, long-maturing Grana Padano cheese, whose experimental results indicate that the AFM_1 concentration levels increased in both curd (3-fold) and cheese (4.5-fold), with similar contamination levels (30-98 ng kg^{-1}) to those encountered in the present work, and with comparable data for curd.

No AFM_1 was detected in the brine used to salt the produced Manchego cheeses, although they were submerged for a short period (8 hours) if compared to other cheeses (3-6 months) which are also placed into brine (Govaris et al., 2001; Oruc et al., 2006).

Yet not all the AFM_1 in milk remains in curd, but may transfer to whey at concentrations of 42.3 % (vat 50) and 51.3 % (vat 100) for the milk originally used in this work. These results fall in the range of 40 % to 60 % as described in the bibliography (Brackett and Marth, 1982; Govaris et al., 2001; Oruc et al., 2006; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009). Besides, Table 2 shows that the total amount of AFM_1 in milk is approximately the sum of the corresponding amounts found in curd and whey, with mean values of 507.4 and 792.8 ng for vat 50 and vat 100, respectively.

Whey presented mean AFM_1 levels (Table 2), which roughly doubled when comparing the two vats: 20.2 ng kg^{-1} vs. 41.2 ng kg^{-1}.

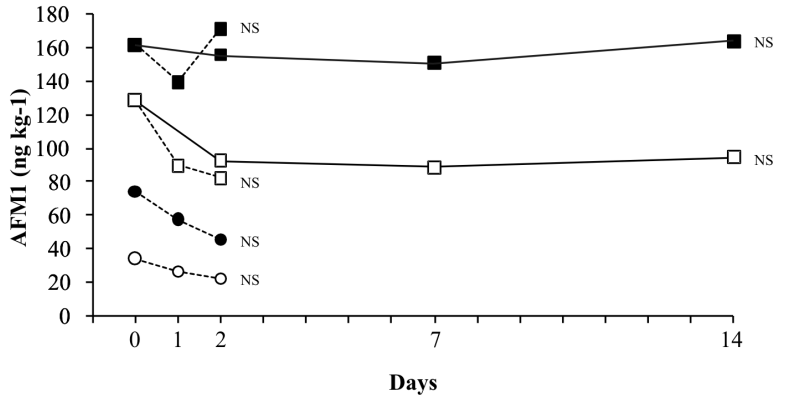
Table 2. Results of the AFM_1 analyses of Manchego cheese products

Sample (n=2)[1]	Vat 50[a]		Vat 100[b]	
	AFM_1[c] (ng kg^{-1})	Total AFM_1 (ng)	AFM_1[c] (ng kg^{-1})	Total AFM_1 (ng)
Milk	47.8±1.9	497.1	80.3±7.1	811.1
Curd	108.0±9.0	377.9	161.7±7.3	504.5
Cheese	139.0±6.4	-[2]	221.3±3.2	-[2]
Whey	20.2±0.5	129.5	41.2±9.6	288.3
Requesón	34.3±5.8	19.2	74.1±6.2	40.8
Requesón whey	16.1±1.4	87.4	35.7±4.7	187.6

[a]Means of two cheese-making procedures using milk contaminated at 50 ng kg^{-1}
[b]Means of two cheese-making procedures using milk contaminated at 100 ng kg^{-1}
[c]Mean±sd
[1]Two samples of each product were analysed
[2]Only part of the curd was transformed into cheese, so the total amount of AFM_1 is not representative

When whey was used to make Requesón, once again, AFM_1 levels were approximately double when comparing vat 50 and vat 100, with a mean concentration of 1.7 times higher than the corresponding whey, which is a much lower value than those found in curd or cheese. A similar situation to whey occurs with Requesón whey; that is, the amount of AFM_1 in vat 100 is twice that of vat 50 (16.1 ng kg^{-1} vs. 35.7 ng kg^{-1}), and represents 33.7 % and 44.4 % of the AFM_1 concentration detected in milk, respectively. Nevertheless, when the total AFM_1 amounts present in Requesón plus Requesón whey were calculated, a mean toxin loss of around 19 % was observed if compared to the corresponding whey they were manufactured from, whose values were 106.6 and 228.4 ng for vat 50 and 100, respectively. This implies that AFM_1 lowered, which could be attributed to the high temperatures reached (90 °C) while producing Requesón, which could alter proteins' structure and low their union with AF (Barbiroli et al., 2007). Yousef and Marth (1989) also previously described this situation for heat treatments in milk and cheeses. In addition, Requesón composition was not a factor that influenced this drop in the different concentrations ($P>0.05$). Requesón production in Spain is an option used to avoid the high costs involved in destroying whey given its high contamination capacity. As it may be used for animal feed or for manufacturing Requesón destined for food consumption, acquiring information about AFM_1 levels in whey is very worthwhile, and it is equally important to consider that approximately 83 % of the amount of AFM1 in whey could be present in Requesón whey.

Sometimes, the preservation of samples before their analysis is needed due to the laboratory work plan, and the best option to do it is using cold treatments (refrigeration or freezing) (Brackett and Marth, 1982; Wiseman and Marth, 1983; Blanco et al., 1988). According to the results of this work, AFM_1 levels would not be affected if these treatments were required to preserve the solid intermediate dairy products, even after 14-day periods (Figure 1), on the same line that several authors, who have studied the stability of this toxin in refrigerated (Rubio et al., 2009) and frozen milk (Stoloff et al., 1975; Rodríguez et al., 2003; Josephs et al., 2005; Rubio et al., 2008) and no effect on concentration has been reported. However, no such studies using curd or Requesón samples have been published. When refrigeration was studied (Figure 1), a slightly lower AFM_1 content was observed in the vat 50 curd and Requesón samples, although no statistical differences ($P>0.05$) among the 3 analysis days were found. A similar situation occurred with the freezing experiment on the first 2 analysis days, when the same values as those obtained for refrigeration with vat 50 curds were noted, and were more evident for vat 100 curds. Afterwards, the toxin concentration remained constant until analysis day 14, thus the cold effect was more marked in the first 48-hour period; once more, no statistically different values ($P>0.05$) were found. Both cold treatments were also studied by Wiseman and Marth (1983), who



*Vat 50 curd (□); vat 100 curd (■); vat 50 Requesón (○); vat 100 Requesón (●); NS - non significant

Figure 1. Evolution of the mean AFM_1 concentrations of the curd and Requesón samples during the refrigeration (----) and freezing (—) periods

used different cheeses made from cow's milk and who encountered no effect on AFM_1 concentration; however, these authors worked with treatments applied to cheese samples and not to curd or Requesón samples as this study does.

Conclusions

The results of this work indicate that when AFM_1 appears in ewe's milk at the EU limit level, or at double this limit, the concentrations resulting in the produced curd and Manchego cheese may present an approximate mean of 2- and 3-fold higher than the initial ones. In the Requesón samples, these mean values were 1.7-fold higher than those in whey. These levels would be considerably below the toxic doses (Tolerable Daily Intake = 2 ng kg^{-1} body weight per day), which poses a health problem if an adult consumed a mean of 100 g of contaminated cheese per day. However, the secondary final products obtained after this manufacturing process (whey, Requesón and Requesón whey) would also contain AFM_1 levels, which should be carefully considered if destined to human or animal consumption. On the other hand, refrigeration or freezing treatments neither affect the preservation of dairy products for appropriate laboratory work planning management purposes nor influence AFM_1 content.

Aflatoksin M_1 u mliječnim međuproduktima nastalim u proizvodnji Manchego sira: razdioba i postojanost

Sažetak

U istraživanju je utvrđivana razdioba aflatoksina M_1 (AFM_1) u grušu, sirutki, Manchego siru, tradicionalnom španjolskom Requesón siru načinjenom od sirutke i Requesón sirutki te njegova stabilnost tijekom dva različita tretmana hlađenja. Svježe ovčje mlijeko je "umjetno" kontaminirano s AFM_1 u završnoj koncentraciji od 50 i 100 ng kg^{-1} i korišteno je u proizvodnji Manchego sira. Koncentracije AFM_1 mjerene su HPLC fluorometrijskom detekcijom nakon potpunog pročišćavanja imunoafinitetnim postupkom. Srednje vrijednosti koncentracija AFM_1 u proizvedenom grušu i Manchego siru bile su oko 2 do 3 puta veće od prvotnog mlijeka od kojeg su načinjeni, a razine toksina zaostale u sirutki bile su 42,3 % i 51,3 % od početne koncentracije. U Requesón uzorcima srednje AFM_1 vrijednosti bile su 1,7 puta veće od onih u uzorcima sirutke, dok su AFM_1 koncentracije 33,7 % i 44,4 % utvrđene u mlijeku, također utvrđene i u odgovarajućim uzorcima Requesón sirutke. Kratka razdoblja hlađenja i zamrzavanja nisu utjecala na razinu toksina u grušu i Requesón uzorcima. U slučaju kada je ovčje mlijeko namijenjeno za proizvodnju Manchego sira kontaminirano s AFM_1 na razini EU-propisa (50 ng kg^{-1}), ili dvostruko većom od te granice, određena koncentracija tog

toksina pojavit će se u prerađevinama, ali će vrijednosti biti znatno niže od toksičnih doza (podnošljive dnevne doze = 2 ng kg^{-1} tjelesne mase dnevno), što predstavlja određeni problem za ljudsko zdravlje.

Ključne riječi: aflatoksin M_1, ovčje mlijeko, gruš, sirutka, sir

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