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Determination of aflatoxin M_1 presence and concentration in Van Herby cheese

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Abstract: Aflatoxins are considered the most toxic secondary metabolites of concern to food safety due to their wide distribution and high toxicity in foods and feeds. The aim of this study is to identify the prevalence of aflatoxin M_1 (AFM_1) in Van Herby cheeses (brined/dry salted). A total of 90 brined and dry salted Van Herby cheese samples offered for retail sale were analysed. The AFM_1 level in the samples was determined by the chromatographic [High-Performance Liquid Chromatography (HPLC)/Fluorescent Detection (FLD)] method. Brined Van Herby cheese samples contained AFM_1 in amounts ranging from < LOD to 0.573 ng·g^{-1} with a mean of 0.165 ± 0.206 ng·g^{-1}, while dry salted Van Herby cheese samples contained < LOD to 0.017 ng·g^{-1} AFM_1. The analysis of the prevalence of AFM_1 in brined and dry salted Van Herby cheese samples was 17.78% (n = 8) and 2.22% (n = 1), respectively. In Van Herby cheese production, standardisation, quality improvement and food safety control procedures need to be used effectively and disseminated. In addition to good agricultural and storage practices to prevent mycotoxin formation, measures must be taken to prevent aflatoxin contamination in animal feed. These applications and systems will provide positive contributions in terms of total quality, nutrients and public health, as well as different advantages such as technological superiority.

Keywords: food safety; HPLC; mycotoxins; public health

Contamination of foods with various biological and chemical toxins poses a major food safety concern worldwide (Campagnollo et al. 2016). Mycotoxins are toxic agents with high toxic activity and stability that are produced by some fungal species in the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, and *Alternaria* during the production, packaging, transportation, and storage of livestock and vegetable foods (Campagnollo et al. 2020). Mycotoxin (aflatoxins, patulin, ochratoxin, etc.) contamination in foods not only causes economic losses, but also endangers human health, industry and food safety (Campagnollo et al. 2016; EFSA 2020).

Aflatoxins are highly toxic fungal metabolites, produced by at least 20 species of three different sections of the *Aspergillus* genus, such as *Flavi*, *Nidulantes* and *Ochraceorosei* (Baranyi et al. 2013; Campagnollo et al. 2016). There are more than 20 species of aflatoxins, and the most prominent ones are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and aflatoxin M_2 (AFM_2). Aflatoxins are acknowledged as the most toxic subordinate metabolites that are of concern for food safety due to their wide dispersion and high toxicity in foods and feeds (Campagnollo et al. 2016;

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Vaz et al. 2020; Gonçalves et al. 2022). AFB_1, which is taken by lactating animals through contaminated diets, is metabolised in the liver and then approximately 1–6.2% is secreted into milk as AFM_1. They can be transferred to dairy products such as cheese and yoghurt, due to its affinity for milk proteins, especially casein, and its stability during heat treatment, storage and processing (Vaz et al. 2022). The presence of AFM_1, which is frequently detected in milk and dairy products, is considered an important public health problem (Ergin et al. 2023). Aflatoxins are a group of mycotoxins with potent toxigenic effects, including immunosuppressive, mutagenic, teratogenic, carcinogenic and hepatotoxic properties, and can induce pernicious health effects (Elsanhoty et al. 2014). The International Agency for Research on Cancer (IARC) classifies aflatoxins as Group 1 (carcinogenic to humans) (IARC 2025). The most notable effect on human health is hepatocellular carcinoma (HCC), which is also acknowledged as the ninth and seventh most prevalent cancer in women and men globally, respectively (Ismail et al. 2018). The presence of AFM_1 in milk and dairy products is undesirable due to its toxicity, and it poses serious concerns for public health due to regular consumption of these products (Vaz et al. 2022). Developed countries and other international organisations have established strict regulations regarding the maximum allowable limits for AFM_1 in milk and dairy products for the purpose of protecting food safety and public health. However, these maximum permitted limits vary depending on the economic conditions, cultural and nutritional habits of the countries. According to the European Commission Regulation [Commission Regulation (EU) 2023/915] and Turkish Food Codex (Official Journal No. 32360, 2023), this limit in milk is 0.05 $\mu g \cdot kg^{-1}$ for consumption by adults, and 0.025 $\mu g \cdot kg^{-1}$ for food products for babies and children. In accordance with the Codex Alimentarius Commission (CAC) recommendation, the United States (US) and many other countries have set a 10 times higher limit (0.5 $\mu \cdot kg^{-1}$) in milk. This legal regulation also aims to ensure that other dairy products, other than drinking milk, such as cheese, are produced from milk suitable for AFM_1 (Vaz et al. 2020). In this context, monitoring AFM_1 concentrations in milk and derivative products can be considered an important part of food safety activities aimed at protecting the health of consumers (Khaneghah et al. 2021).

Generally, various analytical techniques such as High-Performance Liquid Chromatography with Fluorescence Detector (HPLC/FLD) and Enzyme-Linked Immunosorbent Assay (ELISA) are used for quantitative analysis in the identification and quantification of AFM_1 in milk and dairy products. HPLC and Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) methods stand out due to their low measurement limits for quantitative analysis, high recovery rates and method accuracy (EFSA 2020).

Cheese is one of the most consumed dairy-derived products worldwide (Tilocca et al. 2020). Among the many types of cheese produced industrially in Turkey, there are many cheeses produced using traditional methods. Van Herby cheese, a cheese type of Turkey, is a full-fat, semi-hard cheese type that is mostly produced from sheep' s milk, but cow's milk, goat's milk or a mixture of these can also be used. A total of 19 different plant species (*Allium schoenoprasum* L., *Anhriscus nemorosa*, *Ferula orientalis* L., *Mentha spicata*, *Thymus migricus*, *Ferula* sp. and etc.), 6 of which are obligatory and 13 of which are optional, are used, with a maximum rate of 2%. It is produced in two different ways: 'brined' and 'dry salted' (TPTO 2018).

It was aimed to determine the presence/amount of AFM_1 in brined/dry salted Van Herby cheeses and, thus, identify potential risks for consumers by this study. Furthermore, this study aimed to provide scientific data for the assessment of this cheese variety as a quality parameter in terms of sector components and possible legal regulations, as well as the protection of its market and brand equity.

MATERIAL AND METHODS

Collection of samples. This study evaluated a total of 90 (45 brined and 45 dry salted, ~ 90 kg) Van Herby cheese samples, each weighing approximately 1 000 g, produced by traditional methods in Van province (Turkey) in 2020 (spring/summer). Van Herby cheese samples were selected from products supplied to the market at different sales outlets (market, deli, cheesemongers' bazaar) in the centre of Van province in March 2021. The samples were collected under aseptic conditions in sterile sample bags (Whirl-Pak Stand-Up (2 041 mL), USA), delivered to the laboratory under cold chain protection in polyethylene styrofoam boxes, and stored at –20 °C (UCF, 310 SSL, Turkey) until AFM_1 analysis. The AFM_1 analysis of Van Herby cheese samples was done following the methods and procedures (Yoon et al. 2016).

Chemicals and reagents for analysis. Deionised water was obtained via an Ultrapure Water system (Synergy® Water Purification System, Germany). Afla-

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Table 1. High-Performance Liquid Chromatography conditions

HPLC device	Shimadzu prominence (Shimadzu, Japan)
Pump	Shimadzu LC-20 AT series, 2 pieces
Degasser unit	Shimadzu DGU-20A 5
Auto sampler	Shimadzu SIL20AC (refrigerated, 4 °C)
Column oven	Shimadzu CTO-10AS VP (heated)
System control	Shimadzu CBM-20 ALITE
Device software	LC solution 1.12 SP1
Configuration	
Injection amount	50 mL
Flow rate	0.5 mL·min^{-1}
Column	Kinetex C18 (150 × 4.6 mm, 2.6 µm)
Detector	Shimadzu SPD-M20A, FLD detector
Column oven temperature	40 °C
Excitation wavelength (λ_{ex})	360 nm
Emission wavelength (λ_{em})	440 nm
Isocratic mobile phase	acetonitrile + methanol + ultra pure water (17/15/68, *v/v/v*, 100 mL)
Duration	15 min

HPLC – High–Performance Liquid Chromatography

toxin M_1 standard solution (Trilogy® Liquid Standard Aflatoxin M_1 TSL 143-2, 0.5 µg·mL^{-1}, 2 mL, USA), Methanol (Supelco-113351, France), Acetonitrile (Supelco-100030, France), Phosphate Buffered Saline (PBS, P3813-1PAK powder, pH 7.4, to prepare 1 L solution, Merck, Germany), Afla M_1 Test Immunoaffinity column (R-Biopharm Easi-Extract® Aflatoxin RP70/RP71, Scotland), and C18 HPLC column (Phenomenex Kinetex C18, USA) were procured from the relevant producers. The operating conditions of the HPLC/FLD device are summarised in Table 1.

Preparation of AFM_1 stock solutions. Aflatoxin M_1 standard solution [0.5 µg·mL^{-1} (500 ng·mL^{-1})] was diluted 1/20 (*v/v*) with 50 µL AFM_1 Standard + 950 µL acetonitrile / distilled water (10/90, *v/v*) (0.025 µg·mL^{-1}/25 ng·mL^{-1}). Concentrations prepared from this AFM_1 stock solution (0.05, 0.10, 0.20, 0.50, 1.0, and 1.5 ng·mL^{-1}) were loaded into a HPLC/FLD device (six replicates for each concentration) for calibration plotting (Figure 1).

Performance criteria. Table 2 shows the linearity, Relative Standard Deviation% (RSD%), limit of detection (LOD), and limit of quantification (LOQ) values of the analytical method. Recovery and repeatability (RSD_r) were calculated based on the results of post-spike HPLC/FD analysis of blind samples (AFM_1 negative cheese samples) at three different concentrations (0.10, 0.25, and 0.50 µg·kg^{-1}). The Recovery and Repeatability (RSD_r) values of the method that was used for aflatoxin M_1 analysis were in accordance with Turkish Food Codex (Declaration No. 2018/10) and European Commission Regulation [Commission Regulation (EC) No 401/2006], and Table 3 shows these values. The present study showed that the method referred to herein presented RSD_R values ranging between 2.1–12.8% (89.4% ± 11.0% – 96.9% ± 2.0%).

AFM_1 extraction from the samples. 5 g of ground Van Herby cheese sample was transferred to a 100 mL Erlenmeyer, 25 mL of acetonitrile/distilled water (30/20, *v/v*) was added, and it was sonicated for 3 min (Bandelin RK 514H, Germany). This homogenate was taken in falcon tubes and centrifuged at 2 500 rpm for 5 min at 4 °C (Sigma®3-30K, Germany). After centrifugation, 10 mL of super-

Table 2. r^2, RSD%, LOD and LOQ values of aflatoxin M_1 standard solution

Standard	r^2	RSD%	LOD (µg·kg^{-1})	LOQ (µg·kg^{-1})
Aflatoxin M_1	0.99993	3.17838	0.016875	0.051135
$f(x)$	–	2.34369e–006 *x + 0.00312457		

r^2 – coefficient of variation; RSD% – relative standard deviation; LOD – limit of detection; LOQ – limit of quantification

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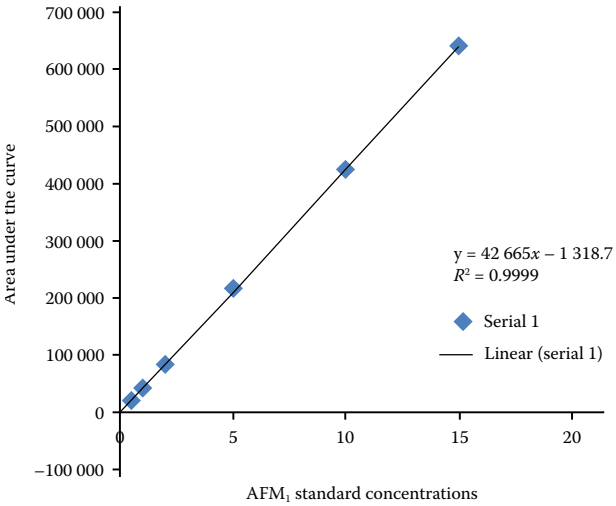

Figure 1. Calibration curve of AFM_1 standard

natant was taken and diluted with 30 mL of PBS (pH 7.2–7.3). The resultant solution was filtered through filter paper (Filter Lab® 1240/90 mm, Spain) into 50 mL special needle syringes, and then this filtrate was loaded onto an Afla M_1 Test Immunoaffinity column (IAC) at 1–3 drops·s^{-1}.

Afla M_1 Test IAC clean-up. After loading, Afla M_1 Test IAC was washed with 10 mL of distilled water (1–3 drops·s^{-1}). Dry air was introduced into the column using an injector and allowed to dry. Finally, Afla M_1 Test IAC was washed with 3 mL of acetonitrile/methanol (30/20, *v/v*), and AFM_1 on the column layer was eluted into a glass tube. The eluate was evaporated to dryness under a stream of nitrogen (N_2) gas at 50 °C. The final residues were dissolved in 0.5 mL of acetonitrile/water (10/90, *v/v*) and passed through a vortex (Heidolph Reax Top D-91126, USA). After filtration through a 0.22 µm millipore filter (ChromXpert® MCE Syringe Tip Filter/PTFE, Germany), the sample was transferred to a vial. All the samples were subjected to the same extraction, IAC loading, and IAC clean up procedures. The prepared vials were loaded into the HPLC/FLD device for AFM_1 analysis. The AFM_1 concentration (ng·g^{-1}) of the samples was calculated using the software integrated into the HPLC/FLD device.

Statistical Analysis. The frequency distributions of AFM_1 identified in the samples in the study and the evaluation of the data were performed with the 2×2 Chi-square test in the SPSS (IBM SPSS 22.0 Ev. Ver) software and the significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

According to Table 4 the incidence (%) and concentrations (ng·g^{-1}) of AFM_1 identified in brined and dry salted Van Herby cheese samples. The analysis results indicated that AFM_1 was identified in eight samples (17.78%, $n = 45$) of brined Van Herby cheese, and only one sample (2.22%, $n = 45$) of dry salted Van Herby cheese (Figure 2). It was determined that 17.78% ($n = 8$) and 2.22% ($n = 1$) of brined and dry salted Van Herby cheese samples were contaminated with AFM_1, respectively (Table 4). AFM_1 incidence of in brined Van Herby cheese samples was statistically significantly ($P < 0.05$) higher than in dry salted Van Herby cheese samples.

Milk and dairy products are among the most basic foods consumed by all age groups all over the world. Aflatoxins have serious toxic effects, especially on infants, children and the elderly. Therefore, contamination of milk and dairy products with AFM_1 is an important public health problem (Madalı and Ayaz 2017). There are data that AFB_1 and AFM_1 exposure is associated with the onset and progression of liver, lung and colon cancer. Recent studies show that aflatoxins increase the formation of reactive oxygen species (ROS) and cause oxidative damage. It is recommended that further studies be conducted to better understand the effects on human health, especially children, who are more susceptible to poisoning due to biological and exposure reasons (FAO/WHO 2018; Marchese et al. 2018; EFSA 2020).

In many studies conducted in Turkey, it was reported that the amount of AFM_1 detected in milk samples exceeded the legal limit (0.050 µg·kg^{-1}) (Mortaş et al. 2022; Commission Regulation (EU) 2023/915;

Table 3. Recovery and repeatability values

AFM_1 level added to samples (Spike rates) (µg·kg^{-1})	Recovery (%, $n = 3$)	Repeatability (RSD_r) (%, $n = 3$)
0.10	81.9 ± 11.6	13.2
0.25	83.1 ± 9.5	10.4
0.50	95.2 ± 12.3	14.5

AFM_1 – aflatoxin M_1, n – High-Performance Liquid Chromatography injection repeat

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Table 4. AFM_1 levels detected in Van Herby cheese samples ($ng \cdot g^{-1}$)

Sample type	Samples total	AFM_1 positive		AFM_1 levels ($ng \cdot g^{-1}$)								
				< 0.016 $\mu g \cdot kg^{-1}$ (< LOD)		≥ 0.016–0.05 $\mu g \cdot kg^{-1}$ (LOD – LOQ)		≥ 0.05 $\mu g \cdot kg^{-1}$ (≥ LOQ)		min.	max.	mean ± SD
	(*n*)	(*n*)	(%)	(*n*)	(%)	(*n*)	(%)	(*n*)	(%)	($ng \cdot g^{-1}$)		($ng \cdot g^{-1}$)
Brine	45	8	17.78	37	82.22	4	8.88	4	8.88	0.016	0.573	0.165 ± 0.206
Dry salted	45	1	2.22	44	97.78	1	2.22	ND	ND	< LOD	0.017	–
Total	90	9	10.00	81	90.00	5	5.55	4	8.88	0.016	0.573	0.165 ± 0.199

AFM_1 – aflatoxin M_1; LOD – limit of detection; LOQ – limit of quantification; ND – not detected; *n* – number of samples

Turkish Food Codex Official Journal No. 32360, 2023). A significant portion of milk production in Turkey is allocated to the cheese production industry, and the consumers include people from all groups. Therefore, cheese production should be inspected and monitored thoroughly for food quality standards and food safety.

The number of brined Van Herby cheese samples that tested negative (< LOD) for AFM_1 was 37 (82.22%) and they contained AFM_1 at the level of a minimum of 0.016 $ng \cdot g^{-1}$, a maximum of 0.573 $ng \cdot g^{-1}$, and the mean of 0.165 ± 0.206 $ng \cdot g^{-1}$. Only one sample among the dry salted Van Herby cheese samples tested positive for AFM_1 and contained toxins at a concentration of 0.017 $ng \cdot g^{-1}$, while 44 (97.78%) samples were AFM_1 negative (< LOD) (Table 4). It is considered that the lower level of AFM_1 in dry salted Van Herby cheese samples compared to samples ripened in brine may be due to the longer ripening period. While brined Van Herby cheeses are offered for consumption after being ripened in plastic containers in a cool place for at least 30 days and at most 60 days, dry salted Van Herby cheeses are offered for consumption/market after being ripened for at least 4 months and at most 7 months (TPTO 2018). A possible reason for the increase in AFM_1 concentration in the early stages of the ripening period may be related to the AFM_1 concentration and moisture loss in cheese during this period (Pecorelli et al. 2019). In addition, enzymatic activities can release AFM_1 bindings and reduce the toxin load in cheese. On the other hand, the reduction of AFM_1 during the storage in some cheeses is attributed to the degradation of the toxin over time (Pecorelli et al. 2018).

Previous studies have shown that the AFM_1 concentrations in sheep's milk and cheeses produced from sheep's milk are lower than those in cow's milk and cheeses produced from cow's milk. It has been suggested that this difference may be attributed to thevarious carry-over index (the ratio between the amount of AFB_1 in consumed feed and the amount of AFM_1 secreted into milk) of the species (Montagna et al. 2008). It has also been reported that feeding sheep and goats with feeds and silages

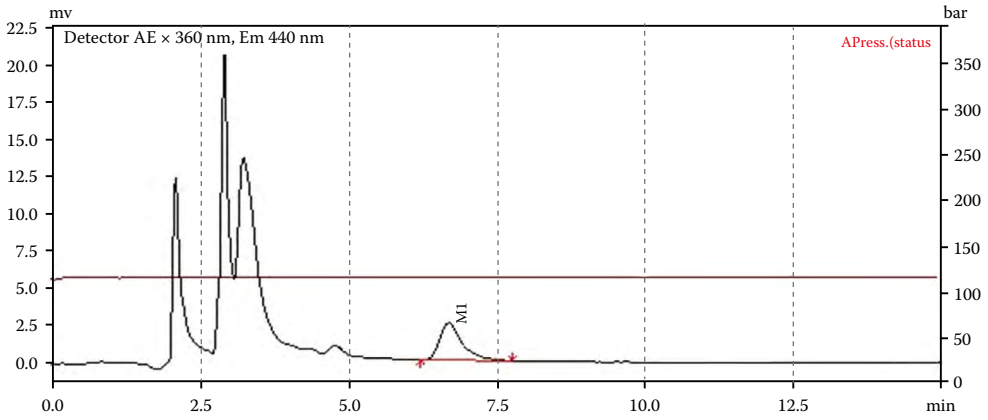

Figure 2. High-Performance Liquid Chromatography (HPLC)/Fluorescent Detection (FLD) chromatogram of sample No. 1

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containing AFB_1 at high concentrations for a shorter time compared to cows may also be effective (Elsanhoty et al. 2014; Campagnollo et al. 2016). Besides these factors, it has been reported that differences in AFB_1 detoxification processes and the digestive systems of different livestock (cows, sheep, and goats) may affect AFM_1 concentration in milk (Anfossi et al. 2012). According to the results of this study, in terms of AFM_1 positive samples, it is evaluated that the sheep from which milk was obtained to produce brined and dry salted Van Herby cheese were fed with feed contaminated with AFB_1 at different levels and/or different amounts of AFM_1 positive cow/goat milk may have been mixed into the sheep milk used in production. Although sheep's milk is frequently used to produce Van Herby cheese in the region, cow's milk, goat's milk, or a combination thereof may also be used (TPTO 2018).

When the previous studies on AFM_1 contamination in cheese varieties were reviewed, similar findings to data of the present study were found (Yaroğlu et al. 2005; Kireçci et al. 2007; Yeşil et al. 2019), as well as analyses including different results (Montagna et al. 2008; Dinçel et al. 2012; Acaröz 2019; Akgül and Kara 2022). Different results may be attributed to different parameters such as year of production, seasonal period, geography, cheese type, toxin concentration in milk used in production, methods used in analyses, extraction techniques, and also possible AFB_1 contamination levels in feeds used in animal feeding. The concentrations of AFM_1 in cheese varieties may diversify depending on various factors such as the type of cheese analysed, cheese production procedures and production technologies, storage conditions (water content eliminated during processing, fermentation temperature, salt concentration, relative humidity, pressing time, cut size, brine, and pH of cheese, etc.) and ripening, seasonal conditions and climate, geographical region, feeding systems of livestock, contamination degree (milk quality), milk used in production (geographical origin of the country, type of milking animal, and seasonal conditions), and analytical methods used in toxin identification. Therefore, it has been reported that there may be significant differences between studies for AFM_1 concentration in cheeses (Anfossi et al. 2012; Campagnollo et al. 2016; Khaneghah et al. 2021).

CONCLUSION

Good Storage Practices (GSP) and Good Agricultural Practices (GAP), monitoring and controlling AFB_1 contamination in animal feed and AFM_1 in milk/dairy products and enforcing regulatory limits are crucial for minimising human exposure to AFM_1. Moreover, more attention to the production, harvesting, drying, storage, etc. of products used as animal feed and the use of modern production techniques such as GAP in the production and storage processes of feed crops will contribute positively to the prevention of AFM_1 contamination in milk/dairy products. Strict control precautions should be taken for AFM_1 contamination in milk used in Van Herby cheese production, and effective use of monitoring procedures should be established to protect public health and reduce economic losses for producers. In order to eliminate possible risks and potential threats to public health, it would be useful to determine legal threshold values for the amount of AFM_1 for other dairy products, including cheese. Furthermore, monitoring AFM_1 levels in milk and dairy products continuously and conducting more comprehensive and active studies will yield significant data.

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