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Enzyme Immunoassay for Mycophenolic Acid in Milk and Cheese

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Mycophenolic acid (MPA) was reacted with *N*-hydroxysuccinimide and conjugated to keyhole limpet hemocyanin (KLH), and to horseradish peroxidase (HRP), respectively. The MPA−KLH was used to produce anti-MPA antiserum in rabbits. A competitive direct enzyme immunoassay (EIA) for MPA was established with anti-MPA antiserum and MPA−HRP conjugate. The mean 50% inhibition and detection limit of MPA standard curves ($n = 103$) were 197 ± 67 and 81 ± 48 pg/mL, respectively. The EIA was specific for MPA and its synthetic 2-morpholinoethyl ester, mycophenolate mofetil (91% relative cross-reactivity). Raw bulk milk and pasteurized milk, with and without β-glucuronidase pretreatment, were analyzed by EIA. No MPA was found in milk, at a detection limit of 100 pg/mL (recovery 58−66% at 0.125−2 ng/mL). Blue-veined cheese from the German market ($n = 53$) was analyzed by EIA, and the detection limit was at 0.5 ng/g (recovery 68−79% at 5−100 ng/g). All but two cheeses contained MPA, although mostly (66%) at levels of <10 ng/g. MPA at 400−1200 ng/g was found in Roquefort cheeses. Highest levels (4−11 μg/g) were found in a German soft cheese preparation. MPA levels in mycelium-rich parts of cheese were 3 times higher than in mycelium-free parts.

KEYWORDS: Mycotoxin; polyclonal antibodies; immunoassay; milk; cheese; roquefort; *Penicillium*

INTRODUCTION

Mycophenolic acid (6-[4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl]-4-methyl-4-hexenoic acid, MPA, **Figure 1**) was probably the first antibiotic that was crystallized from fungal culture and thoroughly described in scientific literature (*1*). This polyketide compound was originally isolated by Bartolomeo Gosio from *Penicillium* culture in the late 19th century and further described (and first named) in 1913 from cultures of *Penicillium stoloniferum* Thom by Alsberg and Black (*2*).

MPA has a long and unique history because of its attributed properties both as a pharmaceutical and as a mycotoxin. MPA has been used for the treatment of noninfectious dermal diseases such as psoriasis vulgaris since the 1970s (*3*) and has been found useful against rheumatoid arthritis (*4*). Like many other *Penicillium* metabolites, MPA was intensively studied and characterized in the 1930s (*5*). It was tested as an antibiotic and was found to be active against some pathogenic bacteria, but was not considered particularly useful (*1*). In other experiments, MPA was shown to possess antiviral (*6*), antiparasitic (*7*), and antitumor (*8*) activities.

However, the most important medical application of MPA is its use as an immunosuppressant drug. The primary indication is post-transplantational treatment, for the prevention of organ and tissue rejection, mostly after renal transplantations (*9*). More recently, the treament of autoimmune disorders such as lupus erythematosus with MPA has been introduced (*10, 11*). In mammals, the primary mode of action of MPA is the specific inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH). Because IMPDH activity is very high in proliferating cells and in tissues with rapidly dividing cell populations, MPA reversibly inhibits T- and B-lymphocyte proliferation, which ultimately results in immunosuppression (*7*). In contrast to other immunosuppressive agents, MPA is not mutagenic (*12*).

To increase oral bioavalability, MPA is therapeutically used as its 2-morpholinoethyl ester prodrug (*13*), known as mycophenolate mofetil (MMF, RS-61443). It is produced under the trade name CellCept by Roche. Once absorbed, MMF is hydrolyzed rapidly to MPA by plasma and tissue esterase, and further metabolized to the MPA-β-glucuronide (*14*). In renal and hepatic allograft recipients, MMF is used on a lifetime basis at doses of 1−3 g per day. Typical adverse effects of long-

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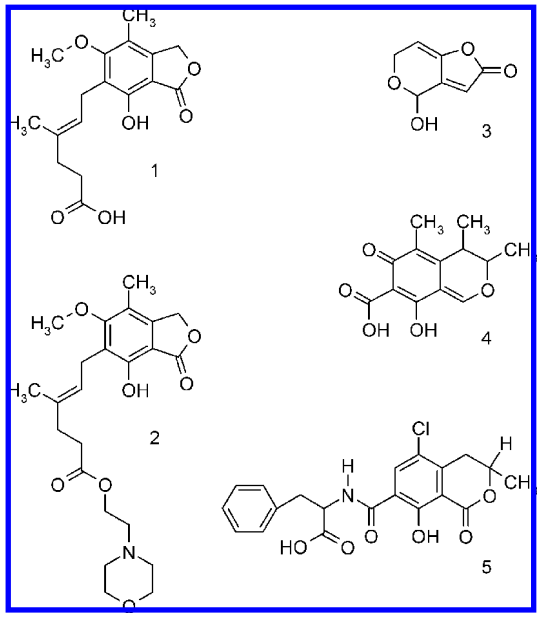

Figure 1. Structures of mycophenolic acid (1, MPA), MPA 2-morpholinoethyl ester (2, MMF), patulin (3), citrinin (4), and ochratoxin A (5).

term treatment with MMF include diarrhea, leukopenia, sepsis, vomiting, and a higher incidence of certain infections (*9, 15*).

Although the acute toxicity of MPA is low (LD_{50}-doses: 700 and 2500 mg/kg, p.os, rat and mouse, respectively), its immunosuppressive effects in humans and in animals are the reason why it is considered as an unwanted naturally occurring mycotoxin in food hygiene and in agricultural science (*2*). The mycotoxin aspect of MPA lies with the fact that the main producer, *P. roqueforti*, is found both in foods and in feeds. The taxonomy of this fungus is somewhat confusing, and the *P. roqueforti* complex has been reclassified into three different species (*16*). Two species (*P. roqueforti*; *P. carneum*) were found to produce MPA, but not the third species, *P. paneum* (*17*).

P. roqueforti is associated with silage; hence MPA is commonly found in feeds. MPA levels as high as 35 mg/kg have been reported for silage (*18, 19*). Little is known about carry-over effects of MPA from silage into milk and tissue of food producing animals. After MPA at levels up to 300 mg/day was fed to sheep, up to 0.23 mg/kg was found in muscle tissue (*20*). In blood serum of these animals, MPA and its glucuronide were detected (*21*). Therefore, carry-over of MPA into milk seems to be possible, although this has not been studied yet.

P. roqueforti is also the most important strain to produce blue-veined cheese, and MPA production has been found in Roquefort cheese and/or in *P. roqueforti* isolated from blue-veined cheeses (*22–26*). However, in most studies published so far, only a very small number of cheese samples has been analyzed, and particularly in older studies analytical methods with relatively poor sensitivity had been used. In 1997, the U.S. Environmental Protection Agency considered *P. roqueforti* as safe in accordance with the toxic substances control act, although some concerns because of its ability to produce mycotoxins under certain fermentation conditions were noted (*27*).

Analytical method development for MPA in clinical medicine focused mostly on liquid chromatography (LC) (*28*), while for food analysis thin-layer chromatography (*22, 23*), LC−UV (*29*), and LC coupled with mass spectrometry (LC−MS) have been described (*26, 30*). Analysis of MPA in human blood, in foods, and in feeds is mostly done by LC−UV, which, except for blood, requires considerable efforts for sample extract preparation. Commercial enzyme-multiplied immunoassays (EMIT, CEDIA) are available for drug monitoring in human plasma (*28, 31*). No information is available concerning the anti-MPA antibody production for these assays. Furthermore, EMIT and CEDIA are exclusively designed for use with specific robotic autoanalyzer equipment in clinical analysis and are as such not available for food analysis. Finally, with detection limits for MPA in serum of 200−500 ng/mL, these tests would not be sensitive enough for food analysis.

Because immunoassays in many cases facilitate the analysis of mycotoxins in food and feed (*32*), here we describe the development and application of a highly sensitive enzyme immunoassay for MPA. Evaluation data for MPA analysis in cows' milk and in blue-veined cheese are presented, and levels of MPA in commercial blue-veined cheeses from the German market are reported.

MATERIALS AND METHODS

Chemicals, Buffers, and Equipment. MPA, patulin, citrinin, and ochratoxin A were from Sigma-Aldrich, Vertriebsgesellschaft, Deisenhofen, Germany. Mycophenolate mofetil (MMF) was a gift from Roche, Basel. Goat antirabbit IgG (whole molecule), β-glucuronidase, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethylformamide (DMF), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), and all other chemicals (reagent grade or better) were also obtained from Sigma. β-Glucuronidase (G-7646) was also from Sigma. Keyhole limpet hemocyanin (KLH, molecular weight used for calculations: 3 000 000) and horseradish peroxidase (HRP, molecular weight 40 000) were from Boehringer, Mannheim, Germany.

The MPA standard was characterized by UV spectrophotometry. Two 10 mL stock solutions (1.0 ± 0.01 mg/mL) of MPA (98% purity) were prepared in methanol, and full scan spectra (190−600 nm) were recorded for diluted solutions in a range from 25 to 100 μg/mL. The UV absorbance maxima for MPA (molecular weight: 320.3) in methanol were at 217 nm, 250 nm ($\varepsilon = 9320 \pm 560$), and 305 nm ($\varepsilon = 4740 \pm 270$). For EIA, working standard solutions were prepared in phosphate buffered saline (PBS; 0.01 M, pH 7.3; phosphate buffer containing 0.1 M NaCl).

The dilution buffer for coating microtiter plates (Maxisorp; Nunc, Wiesbaden, Germany) with antiserum was carbonate−bicarbonate buffer (0.05 M; pH 9.6). To block free protein binding sites of the plates, PBS containing 20 g/L sodium caseinate (Sigma-Aldrich) was used (200 μL per well). The microtiter plate wash solution was distilled water containing 8.5 g/L of NaCl and 0.25 mL/L of Tween 20. Enzyme conjugate dilution buffer was PBS containing 10 g/L sodium caseinate. Enzyme substrate solution (*33*) consisted of 0.2 M potassium citrate buffer (pH 3.9) containing 0.003 M H_2O_2 and 0.001 M TMB (100 μL per well). The enzyme reaction stopping solution was 1 M H_2SO_4 (100 μL per well). Color development for the EIA reaction was measured with an AT 400 microtiter plate reader (SLT, Crailsheim, Germany). The absorbance data were evaluated by an enzyme immunoassay calculation software (*34*) with parameters as described earlier (*35*).

Sample Materials. Raw bulk milk samples ($n = 30$; not homogenized) were obtained from the Bavarian milk control laboratory (Milchprüfring Bayern e.V, Wolnzach, Germany). Pasteurized drinking milk ($n = 25$; homogenized) was purchased from Munich retail shops. Samples of blue-veined cheese ($n = 53$) were purchased from retail shops in the area of Munich, Germany. These samples included products originating from Germany ($n = 14$), France ($n = 21$), Italy ($n = 12$), Denmark ($n = 3$), United Kingdom ($n = 2$), and Spain ($n = 1$). Some of these cheeses were typical blue-veined cheeses with *P. roqueforti* as the sole culture, within the cheese loaf. However, some samples from German producers were labeled as blue-white cheese or blue-red cheeses. The former are prepared with *P. camenberti* as a second culture at the surface of the cheese; the latter type combines *P. roqueforti* inside and bacterial cultures (e.g., *Brevibacterium* spp.) at the outside of the cheese.

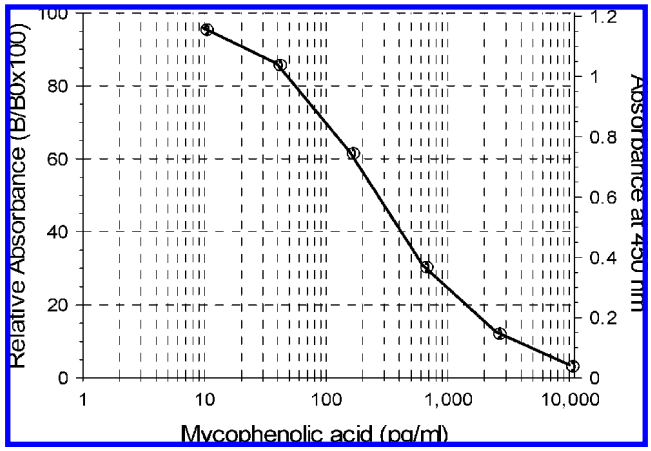

Figure 2. Typical standard curve of the competitive direct EIA for MPA. Four replicate wells of all standard concentrations were analyzed. Coefficients of variation were at 1.5−8%. The absorbance value of the hapten-free blank (B_0) was at 1.26 absorbance units.

Table 1. Standard Curve Parameters of the EIA for MPA (Figure 1) after Evaluation of 103 Standard Curves (MPA in Buffer Solution) Performed Over a Period of 6 months

parameter	50% inhibition concentration	detection limit
mean, pg/mL	197	81
SD, pg/mL	66	48
RSD, %	34	59
min, pg/mL	80	23
max, pg/mL	500	280

Table 2. Recovery of MPA from Artificially Contaminated Pasteurized Milk

	MPA found				
MPA added ng/mL	mean ng/mL	±SD ng/mL	RSD %	recovery %	*n*
0.125	0.07	0.022	31.2	58.3	4
0.25	0.15	0.024	16.1	61.0	6
0.5	0.32	0.043	13.5	63.3	9
1	0.66	0.088	13.3	66.1	13
2	1.30	0.119	9.1	65.2	11

Synthesis of Immunochemicals. MPA was conjugated to KLH and HRP via its carboxyl group using an activated ester method (*36*). MPA (10 mg, 31 μM) was dissolved in 0.5 mL of DMF. NHS (18 mg (155 μM), in 0.25 mL of DMF) and DCC (64 mg (310 μM), in 0.25 mL of DMF) were added and stirred overnight at ambient temperature. KLH (30 mg, 0.01 μM) and HRP (20 mg, 0.5 μM) were each dissolved with 5 mL (for KLH) and 3 mL (for HRP) of a 0.13 M aqueous $NaHCO_3$ solution (pH 8.3). A portion of the activated MPA−NHS intermediate (0.33 mL) was slowly dropped into the KLH solution, another portion (0.15 mL) of the MPA−NHS solution was slowly dropped into the HRP solution, and the conjugation mixtures were stirred at room temperature for 2 h. Next, the MPA−KLH and the MPA−HRP conjugates were separately dialyzed against each three changes (each 5 L) of PBS for 3 days. The conjugates were stored in small portions at −18 °C.

Immunization and Antibody Titer Determination. Animal manipulation has been performed in compliance with the respective German laws and guidelines, and with the formal allowance by the regional Upper Bavarian authority (Regierung von Oberbayern, Sachgebiet 54).

For use as the immunogen, 0.5 mL of MPA−KLH conjugate (approximately 1.5 mg of MPA−KLH) was mixed with 1.0 mL of distilled water and emulsified with 4.5 mL of Freund's complete adjuvant. Rabbits (three female chinchilla bastard, Charles River, Kisslegg, Germany) were each immunized with 2 mL portions of the emulsion by using multisite intradermal injections. A booster injection, using the same composition and amount of immunogen, was given intramuscularly 15 weeks after the primary injection. Blood was collected from the large ear artery. The relative antibody titer was determined in a double antibody solid phase EIA (*37*), using MPA−HRP in a dilution of 1:10 000. Titer was defined as the antiserum dilution that gave 0.3 absorbance units; the preimmune control sera gave maximum absorbance values of <0.1 units under these conditions. The serum of one rabbit collected 21 weeks after primary immunization was used for further characterization.

Table 3. Recovery of MPA From Artificially Contaminated Blue-Veined Cheese

	MPA found				
MPA added ng/g	mean ng/g	±SD ng/g	RSD %	recovery %	*n*
5	3.4	0.79	23	68	13
10	8.2	1.1	13	81	14
20	14.6	2.2	15	73	15
50	35.2	5.7	16	70	14
100	79	15	19	79	16

Competitive Direct EIA. A microtiter plate was coated with antirabbit IgG (10 μg/mL coating buffer, 100 μL per well) overnight at room temperature. Free protein-binding sites of the plates were blocked for 30 min, and then the plate was washed (each well filled three times with wash solution) and made semidry. To each well were added 35 μL of MPA standard solution, 35 μL of MPA−HRP solution (diluted 1:30 000 in 1% sodium caseinate/PBS), and 35 μL of anti-MPA antiserum (diluted 1:100 000 in PBS), and the mixture was incubated for 2 h at room temperature. The plate was washed again, and enzyme substrate solution was added. After 15 min, the enzyme reaction was stopped, and the absorbance at 450 nm was measured. Evaluation of the results by enzyme immunoassay software, calculation of the standard curve parameters (detection limit, 50% inhibition concentration), and calculation of MPA concentrations in extract solutions were performed as described earlier (*35*). In brief, a cubic spline function was used for calculation of the standard curve, and the detection limit (students *t*, 4 replicate wells per concentration, 95% confidence limit) and the 50% inhibition dose were calculated from this standard curve. The measuring range of the standard curve usually was from 20% to 80% relative binding ($B/B_0 \times 100$). Relative assay specificity was tested using standard solutions of MMF, citrinin, ochratoxin A, and patulin at concentrations of up to 10 μg/mL, and comparison of the 50% inhibition concentration relative to that of MPA.

Sample Preparation for EIA Analysis. Milk samples were defatted by centrifugation (1500*g*, 15 min, 4 °C). The skim milk was directly tested by EIA. Another portion of each sample was mixed with aqueous β-glucuronidase solution to obtain a final enzyme concentration of 1000 units/mL skim milk and was incubated at 37 °C for 2 h. For EIA analyses, these two portions of each milk sample were directly used. Further dilutions for EIA analysis were made in PBS if necessary. Recovery was checked by addition of MPA standard solution to pasteurized milk samples before centrifugation.

Cheese samples (5 g) were homogenized with PBS (25 mL) for 5 min in a Stomacher (Kleinfeld Labortechnik, Gehrden, Germany). The homogenate was centrifuged (1500*g*, 15 min, 4 °C), and the aqueous solution was directly used for EIA. Further dilutions were made in PBS if necessary. Recovery was checked using blue-veined cheeses, which were found to contain less than 1 ng of MPA per gram. For routine analysis, a cross-section containing a typical distribution of blue (mycelium) and white (mycelium-free) parts was selected. In a different experiment with some selected cheeses, two portions from the same sample, one being visually free of blue-green fungal mycelium, and another one containing plenty of mycelium, were extracted and analyzed separately.

At least three different dilutions of all milk and cheese extracts were analyzed by EIA, and four replicate wells were analyzed for all standard and extract solutions.

Table 4. Differences in MPA Levels in Blue-Veined Cheeses Depending on the Selection of Test Portions from One Sample, with and without Fungal Mycelium by Visual Inspection

	MPA (μg/kg) in sample parts					
	with mycelium		without mycelium			
cheese	mean	±SD	mean	±SD	ratio[a]	*n*
blue cheese, Germany	3	0.8	1	0.2	3	3
Gorgonzola, Italy	29	0.5	9	0.9	3.2	2
Bavaria blu, Germany	30		11		2.7	1
Roquefort, France	256	64	96	59	2.7	2

[a] MPA (μg/kg) in sample parts with mycelium/MPA (μg/kg) in sample parts without mycelium.

Table 5. MPA in Domestic and Imported Blue-Veined Cheeses from the German Market

			MPA, μg/kg					
country of origin	type of cheese	n/n positive	mean	±SD	25th percentile	median	75th percentile	max
Germany	blue, blue-white, blue-red[a]	10/8	2.4	1.6	1.3	2.6	3.8	4.3
Germany	blue-white[b]	4/4	8240	3200	7700	9100	9600	11 000
France	Roquefort[POD]	9/9	740	260	640	680	820	1200
France	Fourme d'Ambert[POD] and other blue cheeses	12/12	6.4	8.1	1.4	3.1	8.1	28
Italy	Gorgonzola[POD]	12/12	8.3	13.2	1.2	2.7	8.1	46
Denmark	Danablue[POD] and others	3/3	2.0	0.5	1.9	2.2	2.3	2.4
UK	Blue Stilton[POD]	2/2	1.1	0.5	0.9	1.1	1.2	1.4
Spain	Cabrales[POD]	1/1	3.8					

[a] Blue-white cheeses: *P. roqueforti* inside and *P. camenberti* outside. Blue-red cheeses: *P. roqueforti* inside, *Brevibacterium* spp. outside. [b] All four samples were from different lots of the same producer. POD: Protected designation of origin.

RESULTS AND DISCUSSION

Antibodies against MPA. Because MPA has one carboxyl group, it was readily conjugated to carrier molecules via a simple ester formation procedure with NHS. An increased absorbance at wavelengths corresponding to the UV maxima of MPA at 250 nm and at 305 nm relative to the nonconjugated proteins could be detected in the conjugates. Because of an overlap with the absorbance of KLH and HRP at 280 nm, a quantitative determination of the conjugation ratios by UV spectroscopy was not possible. However, the MPA−KLH conjugate induced a high and specific immune response in all three rabbits immunized, with maximum titers of <1:3 000 000. Although all antisera from week 8 onward showed specific inhibition in the pg/mL range, as determined by checkerboard titrations with and without MPA added, the sera of one rabbit were found to give a better sensitivity in competitive direct EIA by a factor of 2−3 as compared to the other two.

EIA Sensitivity and Specificity. Evaluation of standard curve parameters (**Figure 2**, **Table 1**) showed that the mean detection limit was at 81 pg/mL (2.5×10^{-10} M). This is far more sensitive than the commercial MPA immunoassays, which have detection limits in the μg/mL range. The semilinear measurement of the MPA was from approximately 1000 to 100 pg/mL, spanning one log concentration. This was sufficient to determine trace amounts of MPA in sample matrices. When specificity was tested, the 2-morpholinoethyl ester MMF showed strong cross-reactivity. This finding is not surprising, because both the conjugation site of MPA to KLH in the immunogen and the derivatization site of the morpholinoethyl side-chain employ the carboxylic group of MPA. The concentrations required to reach 50% inhibition binding of the MPA−HRP, derived from standard curves established for both compounds, were 197 pg/mL (MPA; 0.61 nM) and 294 pg/mL (MMF; 0.68 nM). Thus, the relative cross-reactivity of MMF is 91%, considering the molecular weight of both compounds (MPA 320, MMF 433). Other mycotoxins, which may co-occur in foods, and sharing some structural similarities (ochratoxin A, citrinin, patulin) did not show competitive binding inhibition in the MPA assay at concentrations up to 10 μg/mL. Because MMF does not occur naturally, the MPA EIA could be regarded as highly specific for MPA when used for food analysis. However, if another, currently unknown, ester derivative of MPA should occur naturally, it most likely would strongly cross-react in the EIA.

Analysis of Milk and Blue-Veined Cheese. The MPA EIA generally was found to be very robust toward sample matrices. Therefore, defatted raw and pasteurized milk, as well as concentrated aqueous cheese extracts, could be analyzed directly, as described for similar EIA applications (*38*). The detection limits for MPA in milk and blue-veined cheese were at 0.1 ng/mL and at 0.5 ng/g, respectively. Average recoveries of MPA were 63% in milk (**Table 2**) and 75% in cheese (**Table 3**).

Because glucuronidation is the major metabolic pathway for MPA (*2*), and because MPA glucuronide was also found in blood serum of sheep after oral administration (*21*), cows' milk samples were analyzed with and without pretreatment with β-glucuronidase. However, none of 30 farm raw bulk milk samples and none of 25 pasteurized milk samples contained detectable amounts of MPA (<100 pg/mL). This part of the study was mainly performed because no information was available whether or not a carry-over of MPA from silage feeds into cows' milk is possible. However, because MPA and its glucuronide were detected in blood serum of sheep after oral administration of MPA, it seems to be reasonable to assume that some carry-over could exist. Besides carry-over, it was shown (*39*) that *P. roqueforti* was one of the frequently isolated fungi from raw cows' milk, with fungal counts of up to 1000 colony forming units, which could imply a theoretical risk of postsecretory MPA production in milk. Our limited results indicate that there is obviously not a major problem with MPA in drinking milk. However, to eventually clarify whether or not there is a carry-over, a MPA feeding study with lactating cows under controlled conditions would be desirable. Furthermore, the implication of fungal contamination of raw milk for raw milk cheese production in aspects of MPA formation should be clarified.

Duplicate analyses of MPA-positive cheese samples in a concentration range from 0.5 to 11 000 μg/kg showed that EIA analysis of diluted aqueous extracts yielded reproducible results, with RSD values of 9.1 ± 10.7%. With a total of 53 samples analyzed, our study also presents the largest data set on MPA in cheese published so far. Nearly all (96%) blue-veined cheeses were found to contain MPA at >0.5 μg/kg; only two samples of blue-white cheese from Germany yielded negative results. The MPA concentration was about 3-fold higher in cheese parts, which were rich in fungal mycelium, as compared to parts of the same sample without visible mycelium (**Table 4**). For routine MPA determination, 5 g of material constituting a cross-section of each sample was therefore analyzed.

There were marked differences concerning the typical levels of MPA found for different brands of blue-veined cheese (**Table 5**). All nine samples representing the "original" Roquefort cheese from France (Protected Designation of Origin, POD) contained considerable amounts of MPA, within a relatively narrow concentration window (740 ± 260 μg/kg; min, 420 μg/kg; max, 1200 μg/kg). All other 12 blue-veined cheeses from France had much lower MPA levels (6.5 ± 8.1 μg/kg). All 12 cheeses from Italy were of the Gorgonzola type, and all contained low levels of MPA (8.3 ± 13 μg/kg). Most cheeses of German production ($n = 10$; blue, blue-white, and blue-red cheeses) also had very low levels of MPA (2.4 ± 1.6 μg/kg). However, one brand of soft cheese with blue mold from one German producer contained exceedingly high levels of MPA (**Table 5**), corresponding with maximum MPA values reported earlier (Lafont et al., 1978; Engel et al., 1982). Four different batches of this product were analyzed, and MPA was determined to be at 8200 ± 3200 μg/kg. A few samples were from Denmark (Danablue, Castello), United Kingdom (blue Stilton cheese), and Spain (Cabrales). All of these contained low levels of MPA.

In our study, we achieved a low detection limit for MPA in cheese; therefore, it is plausible that we found MPA in some types of blue-veined cheese where other studies could not detect this compound. In general, however, our data are consistent with most of the few reports on MPA in blue-veined cheese published earlier. At a detection limit of 10 μg/kg, Lafont et al. (*22*) found that the majority of Roquefort cheeses (21 out of 25) contained MPA at levels of up to 15 000 μg/kg, while Gorgonzola, German, and other blue cheeses were only occasionally positive in a lower concentration range of 10−100 μg/kg. Engel et al. (*23*) reported an even lower contamination frequency in a small survey of Roquefort and other blue-veined cheeses; MPA was only detected in some Roquefort cheese (250−5000 μg/kg). This is not surprising because the thin layer chromatographic method used by these authors had a detection limit of only 75 μg/kg, and in our study most samples of non-Roquefort cheeses had MPA levels lower than 75 μg/kg. A newer study conducted by Kokkonen et al. (*26*) using LC−MS claims a detection limit for MPA of 0.3 μg/kg, although the lowest tested level for recovery studies was at 50 μg/kg. These authors analyzed 11 blue or blue-white cheeses, mostly from Scandinavia, and found MPA only in one out of two samples of French cheese, at a level of 300 μg/kg. In contrast, all samples contained high levels of roquefortine. Lopez-Diaz et al. (*24*) reported qualitative detection of MPA by thin-layer chromatography (detection limit 20 μg/kg) in 4 of 22 Spanish cheeses. Zambonin et al. (*25*) analyzed Danish (Danablu) and Italian (Gorgonzola) samples ($n = 10$) by LC−UV (detection limit 50−100 μg/kg) and found MPA in three samples (100−500 μg/kg). Some differences in the frequency of MPA contamination may also be a result of individual fungal strain characteristics. Several authors consistently have reported that the ability of *P. roqueforti*, isolated from various blue-veined cheeses, to produce MPA in culture varied greatly (*23, 40, 41*).

The total annual per capita consumption of cheese in Germany is at approximately 22 kg, and hard, semihard, and soft cheeses account for 11 kg per person. Although the share of blue-veined cheese within the latter group is not known, it seems reasonable that they account for a maximum of 10%. This estimate would yield an annual consumption of blue-veined cheeses of approximately 1 kg per person, approximately 3 g per day. Considering a median MPA level of 4 ng/g in these cheeses, the daily intake would be 12 ng. Even when using the 90th percentile value found in this study (1000 ng/g), and assuming a high daily consumption of blue-veined cheeses of 100 g, the daily MPA intake would be only 0.1 mg. These intake estimates are several orders of magnitude below the therapeutical dose of 1.5−3 g. If blue-veined cheese would be the only relevant dietary source of MPA, the risk for the consumer seems to be negligible. Finally, neither blue-veined cheese nor *P. roqueforti* culture material have been found to be toxic (*42, 43*), although this fungus produces a variety of other metabolites in addition to MPA (*44*).

In conclusion, we have developed a sensitive immunochemical method, which for the first time enables rapid and easy analysis of MPA in milk and cheese. Because of the high sensitivity of the MPA EIA, which allows replacement of extract purification with extract dilution, it should be easily applicable to other sample materials such as silage. Finally, this EIA has some potential to be used for the monitoring of MPA plasma levels after therapeutical application of MMF in clinical chemistry, or for MPA determination in animal tissue in carry-over studies.

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