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Solid-phase microextraction-high performance liquid chromatography and diode array detection for the determination of mycophenolic acid in cheese

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Abstract

SPME, using a carbowax/templated resin fiber, interfaced with HPLC–UV/DAD has been optimized for the determination of the mycotoxin mycophenolic acid (MPA) in cheese samples. All the parameters influencing the efficiency of the analyte extraction and desorption have been carefully explored. The procedure has been applied to the analysis of blue-cheese samples such as Gorgonzola and Danablu. Samples were subjected to a preliminary short sonication in bicarbonate buffer (0.2 M, pH 9.7); the subsequent SPME was capable of a selective extraction of MPA, characterized by high recovery yields and detection limits of 50 and 100 ppb for Danablu and Gorgonzola, respectively. The present method is faster and simpler than any other existing method for the extraction of MPA from cheese and does not involve the use of toxic organic solvents. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: SPME-HPLC; Mycophenolic acid; Blue-cheese samples

1. Introduction

Cheese is an adequate substrate for mould growth given suitable conditions of temperature and moisture are satisfied. Incidence studies on moulds in a wide variety of cheeses indicate that *Penicillium* species are the most commonly found, particularly on refrigerated cheese (Northolt, Frisvad, & Samson, 1995). Moreover, some strains of *Penicillium roqueforti* are intentionally used as a starter culture for the production of fermented blue cheeses (Engel & Teuber, 1989; Geisen, Cantor, Hansen, Hozapfel, & Jakobsen, 2001). Although there appear to be no reports of toxic effects in humans following consumption of fungal-processed cheese, current scientific interest in mycotoxins has focused increasing attention on the production of toxic metabolites by these *Penicillium* species. A known metabolite of *Penicllium* associated with blue-veined cheeses is mycophenolic acid (MPA) (Engel, Ernst von Milczewski, Prokopek, & Teuber, 1982; Lafont et al., 1979a; Siriwardana & Lafont, 1978). First isolated from *Penicillium brevicompactum* and subsequently from other species (*P. roqueforti, P. viridicatum*, ecc.), mycophenolic acid showed an antibiotic property that made it as a drug in the treatment of psoriasis (Epinette, 1987). MPA is a reversible, non competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) and effectively blocks the de novo pathway of guanosine nucleotide synthesis, which leads to a reduction in DNA synthesis and proliferation of lymphocytes and subsequent immunosuppression (Carter et al., 1969). Furthermore, acute toxicity and mutagenic effects have been described for chickens, mice, rats and rabbits (Engel et al., 1982; Kajiwara et al., 1982).

As far as we know, existing HPLC methods for the determination of MPA are mainly applied to the analysis of urine and serum samples (Hosotsubo et al., 2001; Jones, Taylor, & Johnson, 1998; Na-Bangchang, Supasyndh, Supaporn, Banmairuroi, & Karbwang, 2000; Svensson, Brattstrom, & Sawe, 1999). In fact, analytical procedures for the determination of MPA in cheese are essentially lacking; the few existing papers on this topic (Engel et al., 1982; Lafont et al., 1979b) are all based on liquid–liquid extraction procedures. This methods are, however, intrinsically laborious, time consuming and employ large amounts of organic toxic solvents.

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Solid phase microextraction (SPME), is a solvent free extraction technique widely applied, mainly coupled to GC, to the monitoring of residues of organic compounds in a variety of matrices. It allows simultaneous extraction and pre-concentration of analytes from sample matrix. SPME has been recently interfaced with LC (Chen & Pawliszyn, 1995) in order to extend its range of application also to non-volatile and/or thermally unstable compounds. However SPME/LC applications are still restricted compared to SPME/GC.

In the present study, solid phase microextraction interfaced with LC-UV/DAD has been successfully applied for the first time to the extraction of mycophenolic acid from blue-cheeses.

2. Experimental

2.1. Chemicals

Mycophenolic acid from *Penicillium brevicompactum* (98%) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Stock solution 1 mg/ml of mycophenolic acid was prepared in methanol and stored at 4 °C in the dark. More dilute solutions were prepared just before use in triply distilled water. All organic solvents used (Sigma-Aldrich, Gillingham-Dorset, UK), were HPLC grade. The mobile phase was filtered through a 0.45 μm nylon membrane (Supelco Inc., Supelco Park, Bellefonte, PA, USA) before use. Working solutions for SPME were prepared in phosphate buffer (5 mM, pH 3) before use.

2.2. Apparatus

The SPME–HPLC system consisted of a SCM 1000 vacuum membrane degasser, a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), a SPME interface (Supelco), consisting of a standard six-port Rheodyne valve with a special fiber desorption chamber (total volume: 60 μl), installed in place of the sample loop, and a 5-μm Supelcosil LC-NH_2 column (250×2.1 mm i.d., Supelco). The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and HP 7470A plotter. A HP 3395 laboratory computing integrator directly connected to the photodiode array detector was also used.

2.3. Chromatographic and detection conditions

The mobile phase used was an acetonitrile/methanol/ammonium acetate buffer (50 mM, pH 7) mixture (78:2:20, v/v/v). The flow rate was 0.2 ml min^{-1} and temperature was ambient. Detection wavelength was 254 nm (4 nm band-width) and reference signal was at 550 nm (50 nm band-width). Spectra were acquired in the 220–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity could be checked by the technique of spectra overlaying, after normalization.

2.4. Solid-phase microextraction

Two kinds of silica fibers coated respectively with a 50 μm thick carbowax/templated resin (CW/TPR-100, Supelco) film and a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco) film were employed in this work for comparative studies. A manual SPME device (Supelco) was used to hold the fiber. The SPME device and procedure have been extensively described elsewhere (Arthur et al., 1990; Pawliszyn, 1997). Working solutions were prepared by spiking 5 ml of phosphate buffer (5 mM, pH 3) with different amounts of mycophenolic acid into 7 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out at room temperature at pH 3 for 30 min under magnetic stirring. Mycophenolic acid desorption was performed in static desorption mode by soaking the fiber in an acetonitrile/ammonium acetate buffer (50 mM, pH 7) mixture (80:20, v/v) into the desorption chamber of the interface for 60 s. Then, the valve was changed to the inject position and the fiber was exposed for 20 s to the mobile phase stream.

In order to evaluate percentages of desorption and carryover, the fiber was left in the chamber after each experiment and a second chromatographic run was performed leaving the interface valve in the inject position (dynamic desorption); this operation mode ensured a total desorption of the analyte remained on the fiber.

2.5. Cheese samples

Fermented blue cheese samples (Danablu, $n=5$ and Gorgonzola, $n=5$) were collected from different local markets. Analyses were performed on a single representative aliquot of each sample. Samples (0.5 g) were previously crumbled in a vial; then, 5 ml of potassium bicarbonate buffer (0.2 M, pH 9.7) were added, the resulting mixture sonicated for 30 min and filtered through a grade 3 paper filter (Whatman). Finally the mixture was acidified to pH 3 with 6N HCl and then subjected to SPME.

Recoveries were calculated by spiking cheese samples ($n=3$) with 100 μl of acqueous standard solutions at a concentration level of 1 and 3 ng/μl, corresponding to a cheese concentrations of mycophenolic acid of 200 and 600 ppb, respectively; samples were then left at room temperature overnight and analyzed as described above. The calibration curve in cheese was constructed by spiking cheese samples ($n=3$) with 100 μl of aqueous

standard solutions in the concentration range 0.5–50 ng/μl, corresponding to a concentration range in cheese of 100 ppb–10 ppm.

3. Results and discussion

3.1. Choice of fiber coating material

Preliminary experiments were performed in order to compare the extraction efficiency of carbowax/TPR-100 and PDMS/DVB coated fibers. Both the fibers were able to extract the mycotoxin but the carbowax/TPR-100 coating was capable of the most efficient extraction. In fact, the relative extraction efficiency of carbowax/TPR-100 and PDMS/DVB coated fibres, calculated as peak areas ratio in the relevant LC-UV chromatograms, was 1.47. The carbowax/TPR-100 fiber was then chosen for further experiments.

3.2. Extraction time and temperature

Adsorption times ranging from 5 to 120 min were investigated, both at room temperatures and at 50 °C, in order to establish the equilibration time for analytes partition between the aqueous and the polymer phase. The extraction time profiles were then obtained by plotting the area counts versus the extraction time, as reported in Fig. 1. As apparent, at 50 °C the absolute responses were lower; thus, further experiments were performed at room temperature. The equilibrium was reached after about 50 min at both the temperatures investigated; anyway, as long as the extraction is performed under reproducible conditions, an adsorption time of 30 min revealed a good compromise between sample throughput and peak response.

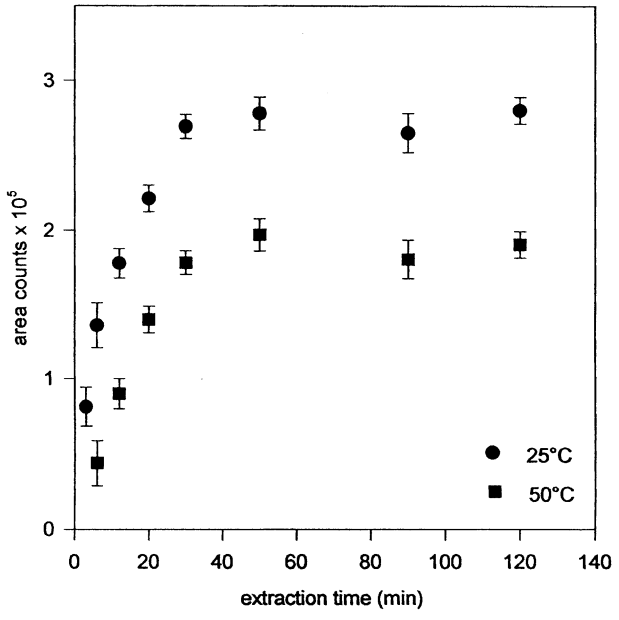

Fig. 1. Area counts versus extraction time obtained at different temperatures.

3.3. Effect of ionic strength and pH

The effect of salt addition on SPME extraction has often been controversial, sometimes increasing the amount of analyte extracted (Bartak & Cap, 1997; Muller, Fattore, & Benfenati, 1997), while showing opposite effects in other cases (Kataoka, Lord, & Pawliszyn, 2000). As shown in Fig. 2, the addition of sodium chloride (ranging from 0 to 200 mg/ml) clearly showed a positive effect in the present case. In any case, it was not considered in the following experiments, since it was found to significantly increase the extraction of interfering compounds during the analysis of real samples. It is worth noting that the positive effect of salt addition on the extraction of MPA could be potentially used in the case of its determination in other matrices.

Fig. 3 reports the effect of pH on the extraction efficiency of mycophenolic acid. As apparent, a significant decrease in the extraction efficiency was observed by varying pH in the range 2.8–5. This was not surprising, since a strong dependence of SPME extraction yield on the pH value was already observed for acidic compounds (Bartak & Cap, 1997; Grote & Levsen, 1999). Then, further experiments were performed at pH 3.

3.4. Desorption conditions and "carry-over"

The dynamic mode was first employed to desorb the analyte from the fiber in the SPME–HPLC interface; this approach produced quantitative recoveries but very broad chromatographic peaks. Thus, the static desorption

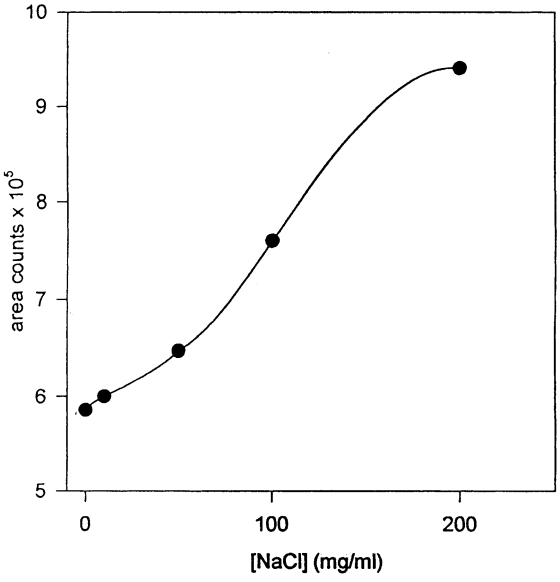

Fig. 2. Effect of salt addition on CPA extraction.

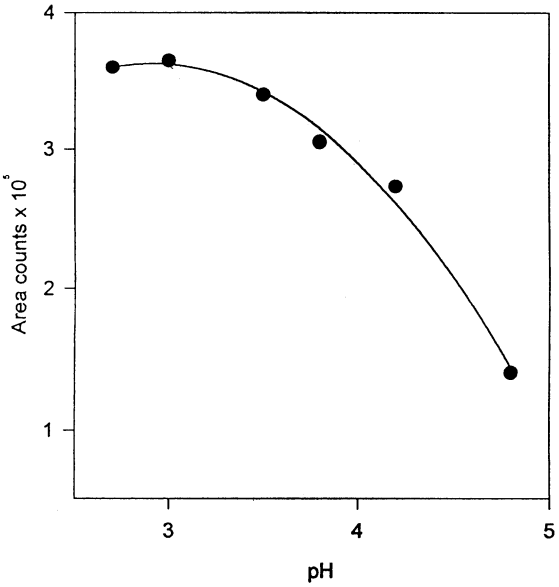

Fig. 3. Effect of pH on CPA extraction.

technique was used for further experiments. The fiber was soaked in mobile phase for a variable period of time before injection into the HPLC column. The best conditions were reached after 60 s of static desorption; then, the fiber was exposed for 20 s to the mobile phase stream. The following step of optimization was the choice of the most efficient desorption solvent; thus, before injection, the fiber was soaked in various acetonitrile/ammonium acetate buffer (50 mM, pH 7) mixtures in the interface, using 60 s of desorption time and switching the valve in the load position 20 s after the injection. The relevant results are shown in Table 1. The best desorption conditions (recovery of 87±3%) were obtained using an acetonitrile/ammonium acetate buffer (50 mM, pH 7) mixture (80:20, v/v).

3.5. Linear range, detection limit and precision

The response of the developed SPME–HPLC procedure was linear in the range 10–500 ppb, with correlation coefficients better than 0.998 and an intercept not significantly different from zero at 95% confidence level.

Table 1
Percentages of desorption and relative standard deviations obtained by varying the composition of the desorption solvent mixture

Desorption solvent	Mycophenolic acid desorbed
Acetonitrile	65±3%
Acetonitrile/ammonium acetate buffer (50 mM, pH 7) (40:60)	62±4%
Acetonitrile/ammonium acetate buffer (50 mM, pH 7) (80:20)	87±3%

The estimated LOD and LOQ obtained on standard solutions were 3 and 10 ppb, respectively, calculated as 3 and 10 fold the standard deviation of the intercept of the calibration curve (Long & Winefordner, 1983).

The ANOVA test shows that variance is independent from the concentration of standard solutions. The within-day precision of the method was investigated on MPA standard solutions at a concentration level of 100 ng/ml by performing daily six replicates. The same solutions were analyzed four times each day for a period of five days for the day-to-day precision evaluation. The within day RSD% and day-to-day RSD% were 4.0 and 7.6%, respectively.

3.6. Cheese samples

Once the ideal extraction and desorption conditions were established, the procedure was applied to the analysis of cheese samples. A short sonication step simply using bicarbonate buffer was performed before SPME on cheese samples treated as described in the Section 2. Fig. 4A and B, shows the SPME–HPLC–UV chromatograms relevant to the analysis of a gorgonzola cheese

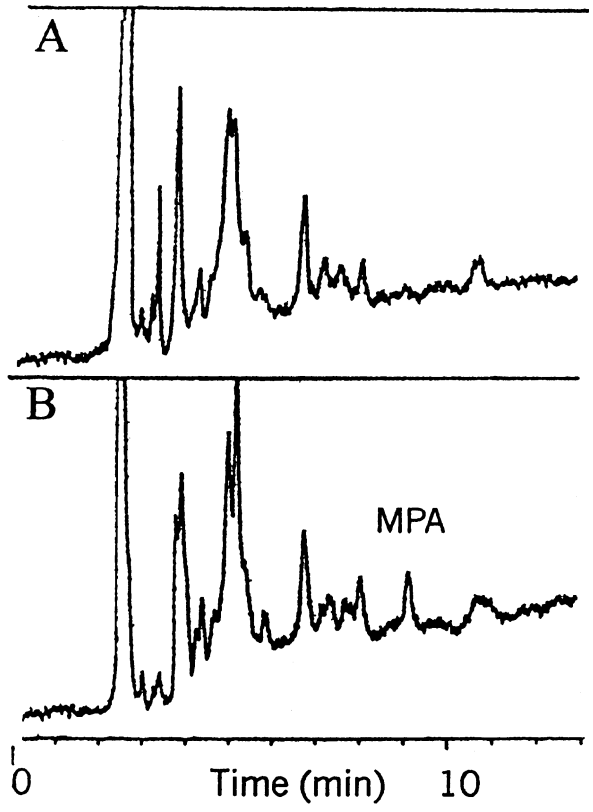

Fig. 4. (A) HPLC–UV chromatogram relevant to the SPME of a cheese extract. (B) HPLC–UV chromatogram relevant to the SPME of the same cheese extract spiked with MPA at its LOD concentration. Attenuation, 4 mAU full scale. Chromatographic conditions and spectral acquisition as specified in the experimental section; detection at 254 nm.

sample unspiked and spiked with MPA at its LOD concentration, respectively. As clearly shown, no occurrence of matrix components was observed at the elution time of MPA (see Fig. 4A) that appears completely separated by interfering peaks (see Fig. 4B). This was a clear indication of the highly selective extraction performed by the employed technique. Percent recoveries, calculated by spiking cheese samples ($n=3$) with mycophenolic acid as described in Section 2, are reported in Table 2. Detection limits obtained were 50 and 100 ppb for Danablu and Gorgonzola, respectively and were calculated according to IUPAC (Long & Winefordner, 1983).

Table 2
Percentage of recovery and relative standard deviations obtained for the extraction of MPA from cheese samples

MPA concentration spiked (ppb)	% Recovery Danablu ($n=3$)	% Recovery Gorgonzola ($n=3$)
200	94±5%	55±5%
600	85±4%	50±4%

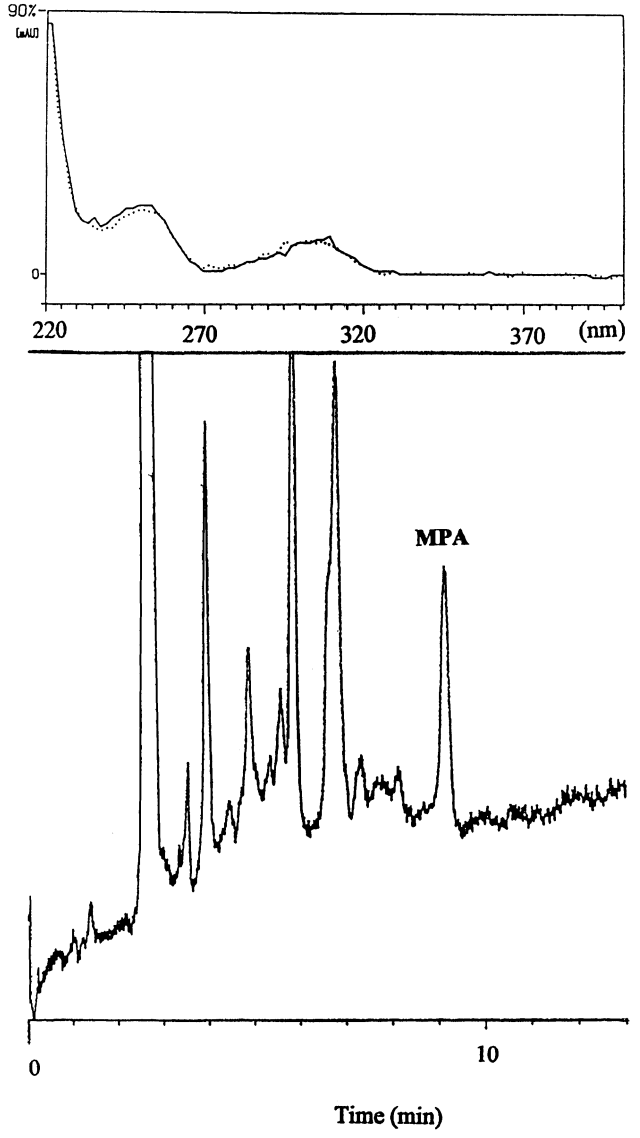

Fig. 5. SPME–HPLC–UV chromatogram relevant to the analysis of a naturally contaminated cheese sample (the inset reports the UV spectrum relevant to the peak of MPA). Attenuation, 4 mAU full scale. Chromatographic conditions and spectral acquisition as specified in the experimental section; detection at 254 nm.

Since a variable and often remarkable matrix effect was observed, a calibration curve in cheese was constructed; it resulted linear for at least two concentration decades, with correlation coefficients better than 0.993 and intercept not significantly different from zero at 95% confidence level.

Three analyzed samples were found to be contaminated with variable quantities of mycophenolic acid. Fig. 5 shows an SPME–HPLC–UV chromatogram relevant to the analysis of a naturally contaminated cheese sample (the inset reports the UV spectrum relevant to the peak of MPA). The estimated concentration of mycophenolic acid in the contaminated samples ranged from 100 to 500 ppb. The RSD% calculated by replicate analysis ($n=3$) on the same cheese sample was 4.5.

4. Conclusions

In this paper, an SPME procedure coupled with HPLC–UV, using a carbowax/templated resin fiber, for the determination of mycophenolic acid in cheese samples has been developed for the first time. The method is simple, highly sensitive and solvent-free. After a short sample sonication step in bicarbonate buffer (0.2 M, pH 9.7) characterized by satisfactorily high recoveries of MPA, samples were subjected to SPME that was capable of a selective extraction. The whole extraction resulted by far simpler and faster than any other existing procedure for mycophenolic acid extraction from cheese and could be potentially applied to the determination of the mycotoxin in other matrices (such as blood). Work in this direction is currently in progress.

References

Arthur, C. L., & Pawliszyn, J. (1990). Solid phase microextraction with thermal disorption using fused optical fibres. *Analytical Chemistry*, *62*, 2145–2148.

Bartak, P., & Cap, L. (1997). Determination of phenols by solid-phase microextraction. *Journal of Chromatography A*, *767*, 171–175.

Carter, S. B., Franklin, T. J., Jones, D. F., Leonard, B. J., Mills, S. D., Turner, R. W., & Turner, W. B. (1969). Mycophenolic acid: an anti-cancer compound with unusual properties. *Nature*, *223*, 23 848–850.

Chen, J., & Pawliszyn, J. B. (1995). Solid phase microextraction coupled to high performance liquid chromatography. *Analytical Chemistry*, *67*, 2530.

Engel, G., Ernst von Milczewski, K., Prokopek, D., & Teuber, M.

(1982). Strain-specific Synthesis of mycophenolic acid by Penicillium roqueforti in blue-veined cheese. *Applied and Environmental Microbiology*, *43*, 1034–1040.

Engel, G., & Teuber, M. (1989). Toxic metabolites from fungal cheese starter cultures (*Penicillium camemberti* and *Penicillium roqueforti*). In H. P. Van Egmond (Ed.), *Mycotoxins in dairy products* (pp. 163–259). London: Elsevier Applied Science.

Epinette, W. W., Parker, C. M., Jones, E. L., & Greist, M. C. (1987). Micophenolic acid for psoriasis. A review of pharmacology, long-term efficacy, and safety. *Journal of the American Academy of Dermatology*, *17*, 962–971.

Geisen, R., Cantor, M. D., Hansen, T. K., Hozapfel, W. H., & Jakobsen, M. (2001). Characterization of *Penicillium roqueforti* strains used as cheese starter cultures by RAPD typing. *International Journal of Food Microbiology*, *65*, 183–191.

Grote, C., & Levsen, K. (1999). The application of SPME in water analysis. In J. Pawliszyn (Ed.), *Application of SPME* (pp. 169–187). Cambridge: Royal Society of Chemistry.

Hosotsubo, H., Takahara, S., Kokado, Y., Permpongkosol, S., Wang, J. D., Tanaka, T., Matsumiya, K., Kitamura, M., Okuyama, A., & Sugimoto, H. (2001). Rapid and simple determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection. *Journal of Pharmaceutical & Biomedical Analysis*, *24*, 555–560.

Jones, C. E., Taylor, P. J., & Johnson, A. G. (1998). High performance liquid chromatography determination of mycophenolic acid and its glucuronide metabolite in human plasma. *Journal of Chromatography B: Biomedical Applications*, *708*, 229–234.

Kajiwara, Y, Kimura, T., Kanbara, T., Kuriyama, T., Naito, Y., Yoshida, S., Akaboshi, S., & Ito, R. (1982), *Res. Lab.*, Ohta Pharm. Co., Ltd., Kawaguchi, Japan, 29,3, 400–411.

Kataoka, H., Lord, H. L., & Pawliszyn, J. (2000). Applications of solid-phase microextraction in food analysis. *Journal of Chromatography A*, *880*, 35–62.

Lafont, P., Debeaupuis, J. P., Gaillardin, M., & Payen, J. (1979a). Production of mycophenolic acid by Pennicillium roqueforti strains. *Applied Environmental Microbiology*, *47*, 365–368.

Lafont, P., Siriwardana, M. G., Combemale, I., & Lafont, J. (1979b). Mycophenolic acid in marketed cheese. *Food Cosmet. Toxicol.*, *17*, 147–149.

Long, G. L., & Winefordner, J. D. (1983). Limit of detection. *Analytical Chemistry*, *55*, 712A–724A.

Muller, L., Fattore, E., & Benfenati, E. (1997). Determination of aromatic amines by solid-phase microextraction and gas chromatography-mass spectrometry in water samples. *Journal of Chromatography A*, *791*, 221–230.

Na-Bangchang, K., Supasyndh, O., Supaporn, T., Banmairuroi, V., & Karbwang, J. (2000). Simple and sensitive high performance liquid chromatography method for the determination of mycophenolic acid in plasma. *Journal of Chromatography B: Biomedical Applications*, *738*, 169–173.

Northolt, M. D., Frisvad, J. C., & Samson, R. A. (1995). In R. A. Samson, E. S. Hoekstra, & Oorschot, Introduction to sùfood-borne fungi. Cantraalbureau voor Schimmelcutures CAN (pp. 243–250). BAARN

Pawliszyn, J. (1997). *Solid phase microextraction theory and practice*. New York: Wiley-VCH.

Siriwardana, M. G., & Lafont, P. (1979). Determination of mycophenolic acid, penicillic acid, patulin, sterigmatocystin and aflatoxins in cheese. *J. Dairy*, *62*, 1145–1148.

Svensson, J. O., Brattstrom, C., & Sawe, J. (1999). A simple HPLC method for the determination of mycophenolic acid and mycophenolic acid glucuronide in plasma. *Therapeutic Drug Monitoring*, *21*, 322–324.