Guenther, E., *Perfum. Essent. Oil Rec.* **59**, 642 (1968).
Mon, T. R., Forrey, R. R., Teranishi, R., *J. Gas Chromatogr.* **5**, 497 (1967).
Munz, P. A., "A California Flora", University of California Press, Berkeley, Calif., 1959, p 693.
Nickerson, G. B., Likens, S. T., *J. Chromatogr.* **21**, 1 (1966).
Oh, H. K., Jones, M. B., Longhurst, W. M., *Appl. Microbiol.* **16**, 39 (1968).
Oh, H. K., Sakai, T., Jones, M. B., Longhurst, W. M., *Appl. Microbiol.* **15**, 777 (1967).

Schuyten, H. A., Weaver, J. W., Reid, J. D., *J. Am. Chem. Soc.* **69**, 2110 (1947).
Teranishi, R., Buttery, R. G., Lundin, R. E., *Anal. Chem.* **34**, 1033 (1962).

Analysis of Blue Cheese for Roquefortine and Other Alkaloids from *Penicillium roqueforti*

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A method has been developed for the analysis of blue cheese containing internal mold for the neurotoxin roquefortine and isofumigaclavines A and B, three alkaloids produced by *Penicillium roqueforti*. Detection was by thin-layer chromatography and semiquantitative estimations were made for roquefortine and isofumigaclavine A. Recoveries of roquefortine added to cheese were 54–66%. Roquefortine was detected in 16 out of 16 samples of blue cheese originating from 7 countries, in estimated concentrations of up to 6.8 ppm. It was usually accompanied by isofumigaclavine A (up to an estimated 4.7 ppm) and traces of isofumigaclavine B were also detected in a few samples. Little or no migration of roquefortine into visibly nonmoldy areas of blue cheese was observed.

Penicillium roqueforti is a fungal species of particular interest to the agricultural and food scientist. Not only is it one of the commonly occurring microorganisms in fermenting silage (Le Bars and Escoula, 1974; Raper and Thom, 1968), including samples associated with mycotoxicoses (Kanota, 1970; Still et al., 1972), but it is also the essential fungus used in the production of Roquefort cheese and other varieties of blue cheese containing internal mold.

Compounds isolated from *P. roqueforti* culture media include "PR toxin", a sesquiterpenoid metabolite (Wei et al., 1973, 1975), and three incompletely characterized substances of unknown structure designated toxins-1, -2, and -3 (Kanota, 1970). The ability of *P. roqueforti* to produce alkaloids was shown by Taber and Vining (1958), Abe et al. (1967), and Bekmakhanova (1974). Only recently, however, have crystalline alkaloids been isolated and characterized. Ohmomo et al. (1975) isolated the known compound festuclavine and two other clavine alkaloids named roquefortine A (the major alkaloid) and roquefortine B, for which the unusual structures 7-acetoxy-6,9-dimethylergoline and 6,9-dimethylergolin-7-ol, respectively, were proposed. A fourth alkaloid, roquefortine C, was not structurally characterized. Scott et al. (1976) obtained two alkaloids from the mycelium of a strain of *P. roqueforti* used in cheese processing. The major alkaloid, designated roquefortine, was assigned the structure 10b-(1,1-dimethyl-2-propenyl)-3-(imidazol-4-ylmethylene)-5a,10b,11,11a-tetrahydro-2*H*-pyrazino-[1',2':1,5]pyrrolo[2,3-*b*]indole-1,4-(3*H*,6*H*)-dione and had physical properties similar to those published for roquefortine C. The minor alkaloid, isofumigaclavine A

(9-acetoxy-6,8-dimethylergoline), was a stereoisomer of fumigaclavine A (Spilsbury and Wilkinson, 1961). Most of the physical properties reported for roquefortine A (Ohmomo et al., 1975) are similar to those of isofumigaclavine A, and they may in fact be the same compound. However, isofumigaclavine B (6,8-dimethylergolin-9-ol), obtained by hydrolysis of isofumigaclavine A (Scott et al., 1976), had a higher melting point than that reported for roquefortine B. Roquefortine is a neurotoxin, provoking convulsive seizures in mice (Frayssinet and Lafarge-Frayssinet, 1975; Scott et al., 1976). It was therefore important to determine whether it occurred in blue cheese and the approximate amounts that could be found in commercial samples. A semiquantitative thin-layer chromatographic method for the analysis of roquefortine in cheese was developed that also allowed detection of isofumigaclavines A and B.

EXPERIMENTAL SECTION

Extraction and Cleanup. Fifty grams of cheese was blended in a Waring blender with 50 ml of chloroform, 100 ml of methanol, and 20 ml of water for 2 min at high speed and then for two further 1-min periods after successive additions of 50 ml of chloroform and 50 ml of water, following the procedure of Shih and Marth (1971) for extraction of aflatoxins from cheese. The mixture was filtered under reduced pressure through a pad of Celite filter aid (AW/545) together with ca. 10 ml of chloroform used to rinse the blender jar. The filter cake was rinsed with a further 10–15 ml of chloroform. The chloroform layer was separated and evaporated on a steam bath under a gentle stream of nitrogen to an oily residue, which was dissolved in 50 ml of ethyl acetate and extracted with two 50-ml portions of 0.5 N hydrochloric acid. The combined acid layers were washed with 50 ml of *n*-hexane, made alkaline with ca. 10 ml of 28% ammonium hydroxide solution, and reextracted with 50 ml of chloroform. The

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Table I. Estimated Concentrations[a] of *Penicillium roqueforti* **Alkaloids in Blue Cheese Samples**

Cheese		Roquefortine, ppm	Isofumigaclavine A, ppm	Isofumigaclavine B
Origin	Sample[b]			
Denmark	A-1[c]	0.87[d,e]	1.7[d-f]	Trace[e]
Denmark	A-2	0.64, 0.67, 1.1	3.1,[d] 2.8, 2.7[d]	Traces
Denmark	A-2, "mold-free"	0.06	2.1	Trace
Denmark	A-2, "high-mold"	1.7	4.7	Trace
Denmark	B[c]	0.45[d]	1.6[d,f]	ND[g]
Denmark	C	2.3, 2.5, 1.6	0.37, 0.37, 0.31	ND
Denmark	D	0.87, 0.87	0.12, 0.15	ND
England (Stilton)	1[c]	3.4[d,e]	Trace ?	ND
England (Stilton)	2	1.3, 1.0, 0.62	0.09, 0.06, 0.09	ND
Finland	1	0.06, 0.09, 0.05	1.8, 1.6, 2.1[e]	Traces[e]
W. Germany	A	0.31, 0.44, 0.37	Traces	ND
W. Germany	A, "mold-free"	ND	ND	ND
W. Germany	A, "high-mold"	6.8[e]	ND	ND
W. Germany	B	0.75, 1.0, 0.75	0.12, 0.12, 0.09	ND
W. Germany	B, "mold-free"	ND	0.02	ND
W. Germany	B, "high-mold"	2.0	0.17	ND
Italy (Gorgonzola)	A	0.16, 0.14	ND	ND
Italy (Gorgonzola)	B	0.16, 0.22	ND	ND
Quebec	A	0.67,[c-e] 1.5	0.92,[c,f] 0.65	Traces[c]
Roquefort	A[c]	0.23[d]	ND	ND
Roquefort	B	0.06, 0.06	0.15, 0.11	ND
Roquefort	C	0.40	0.10	ND

[a] Uncorrected for recovery. [b] Capital letters denote different brands. [c] Initial chloroform evaporation and second acid extraction steps omitted from analytical method. [d] Identity confirmed by uv spectroscopy. [e] Identity confirmed by mass spectrometry. [f] Estimated by uv spectroscopy. [g] Not detected (if applicable, to all subsamples on same line).

extract was evaporated nearly to dryness under nitrogen, transferred to a 16-ml vial for complete evaporation, and dissolved in 0.5 ml (or other suitable volume) of chloroform.

Thin-Layer Chromatography. Silica gel F 1500/ LS254 thin-layer sheets (Schleicher & Schuell) were used for analytical and preparative thin-layer chromatography (TLC) of cheese extracts. Other precoated silica gel layers were also used in a few cases for preparative TLC only. One to 10 μl of extract was spotted for analysis. Chromatograms were developed for a distance of 13–15 cm with chloroform–methanol–28% ammonium hydroxide (90:10:1, v/v/v; CMN) in a lined, equilibrated tank (27.5 × 7.5 × 24 cm inside dimensions); the solvents were replaced at least every 2 days. Chloroform–methanol (9:1, v/v; CM) and chloroform–redistilled diethylamine (8:2, v/v; CD) were used as confirmatory solvent systems (unlined tanks). Analytical TLC plates were sprayed with 50% sulfuric acid and heated at 110 °C for 10 min. Roquefortine formed a light blue-gray spot and isofumigaclavines A and B appeared as mauve spots: typical R_f values were, respectively, 0.41, 0.63, and 0.31 in solvent system CMN; 0.40, 0.44, and 0.04 in system CM; and 0.34, 0.81, and 0.60 in system CD. Amounts of roquefortine and isofumigaclavine A were estimated approximately by visually comparing intensities of the extract spots with those of varying amounts of the corresponding standards developed on the same plate. The alkaloids were located on preparative thin-layer chromatograms by quenching under short-wave ultraviolet (uv) light and, if necessary, by spraying a thin strip at the edge of the plate with 50% sulfuric acid, then eluted from the silica gel with chloroform–methanol (1:1). Preparative TLC was repeated, if required, using a different solvent system to obtain samples of sufficient purity for mass spectral confirmation. Standard solutions of roquefortine (125 μg/ml) and isofumigaclavine A (62 μg/ml) in chloroform were prepared from crystalline metabolites of *Penicillium roqueforti* and stored in the freezer. Isofu-

migaclavine B standard was qualitative only.

Ultraviolet Spectroscopy. Spectra of roquefortine and isofumigaclavine A isolated from cheese extracts were recorded in 95% ethanol with a Unicam SP.800 B spectrophotometer. Estimations of roquefortine were made using ϵ 24 620 at 326 nm and of isofumigaclavine A using ϵ 6456 at 283 nm. A slight correction was necessary with the latter compound to allow for the contribution of the silica gel blank. Experiments were carried out to determine whether changes occurred in the uv spectra of pure roquefortine in 95% ethanol (18.4 μg/ml) and chloroform (16.7 μg/ml) solutions kept in flint glass vials for up to 9 days at room temperature in the dark, on the laboratory bench 200 cm below fluorescent lighting, and ca. 10 cm from a window (western exposure).

Mass Spectrometry. Mass spectra were recorded using a Varian MAT 311 spectrometer at 70 eV and an ion source temperature of 225 °C; probe temperatures were 30–80 °C for isofumigaclavine A, 122–135 °C for isofumigaclavine B, 155–173 °C for roquefortine, and 200 °C for the roquefortine photoproduct. More volatile impurities were removed from TLC samples at lower probe temperatures.

RESULTS AND DISCUSSION

The method of analysis developed for the *Penicillium roqueforti* alkaloids in cheese afforded clean extracts with ready detection of roquefortine and isofumigaclavines A and B, except in the case of certain cheese samples (from Italy, Finland, and Quebec; Table I) which had a faint red spot at the R_f of roquefortine in solvent system CMN. Roquefortine was therefore estimated in the two Italian cheeses, which contained no isofumigaclavine A, using solvent system CM, and in the two other cheese samples using system CMN following its isolation by preparative TLC (solvent system CD).

Direct extraction of the initial chloroform extract with the acid sometimes resulted in emulsions and it was found preferable to carry out this transfer from ethyl acetate
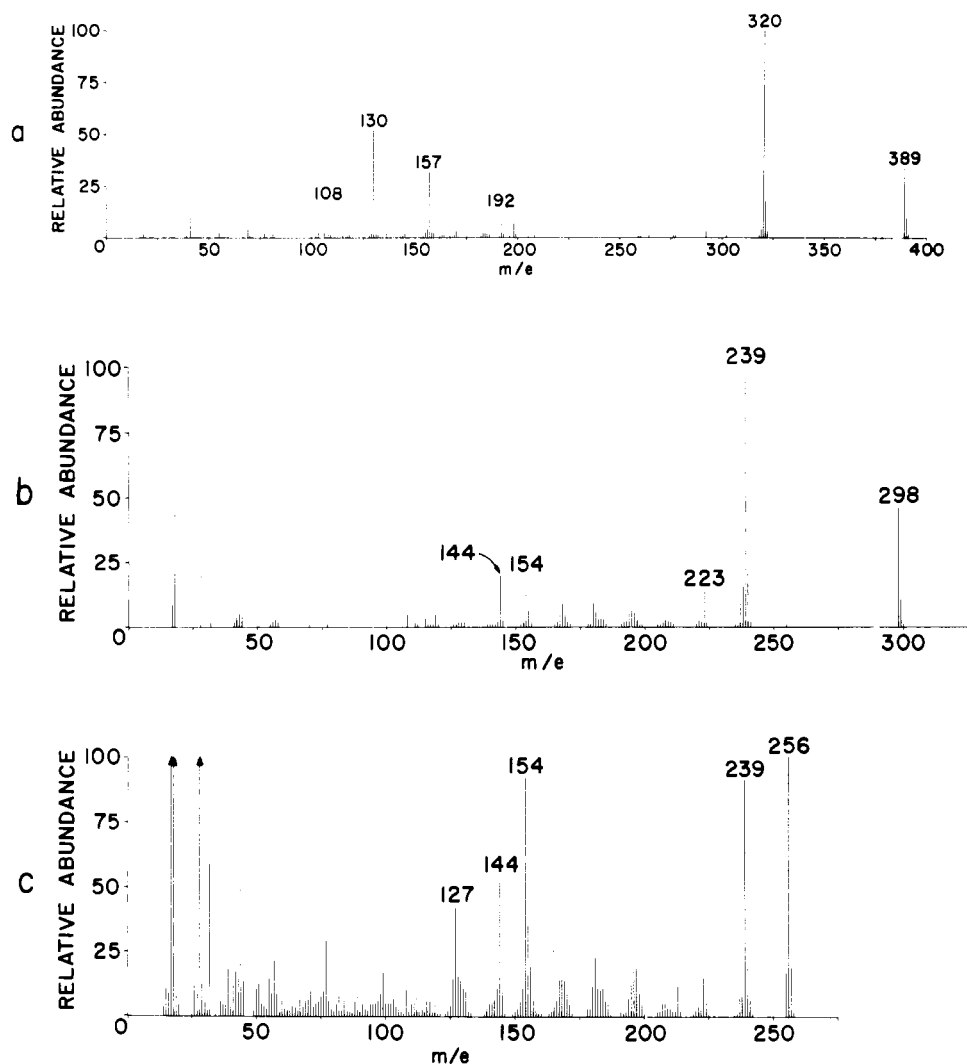
Figure 1. Mass spectra of (a) roquefortine; (b) isofumigaclavine A; (c) isofumigaclavine B.

solution. The hexane wash of the hydrochloric acid extract removed most of the residual lipid materials that otherwise could interfere with detection of isofumigaclavine A.

Although the TLC estimations were only semiquantitative, an assessment of the overall efficiency of the method was made by adding pure roquefortine (500 μg/ml in chloroform) to fresh goat's milk cheese at levels of 0.5, 1.0, 2.0, and 3.0 ppm. The mean recovery of roquefortine was 58.8% (seven experiments, range 54–66%). These recoveries were checked in five cases by uv measurements of roquefortine isolated by preparative TLC and a mean of 50.8% was found (range 42.6–56.0%). Recovery of roquefortine added to one German blue cheese (sample A, Table I), which was taken from the end of the block, carefully trimmed of mold, and found to be free of the alkaloids, was estimated to be 65% by TLC (65% by uv). Isofumigaclavine A (1.69 ppm) added to goat's milk cheese was recovered in an estimated yield by TLC of 45% in one experiment.

Experiments were carried out to try and determine in which steps of the method alkaloid losses might occur. Reextraction of the goat's milk cheese/Celite filter cake yielded an additional 13% roquefortine and 9.6% isofumigaclavine A. These values were in approximate agreement with those obtained with Danish blue cheese samples when different fractions were analyzed separately. Approximately 9.3 and 13% of the sum total of roquefortine found (1.8 and 0.77 ppm, respectively) and 7.5 and 13% of the total isofumigaclavine A (3.9 and 2.8 ppm,

respectively) were recovered on reextraction of two cheese samples. No roquefortine was detected in a second chloroform extract (after cleanup) of the methanol–water phase, a third acid extraction of an ethyl acetate extract, the hexane extract, or a second chloroform extract of the basified acid solution; the first two of these extracts removed additional small amounts (2–6%) of isofumigaclavine A. A significant part of isofumigaclavine B, if present, remained in the methanol–water phase. However, this appears to be a minor component of blue cheese alkaloids and no attempt was made at quantitation.

Dilute roquefortine solutions exposed to daylight in glass vials showed marked changes in uv spectra within 1 day. In 95% ethanol, the absorption maximum at 327 nm shifted to 314 nm and after 9 days was at 312 nm, while the maxima at 242 and 210 nm were unchanged. In chloroform solutions, roquefortine had an absorption maximum at 337 nm (ϵ 24 986), which changed after 1 day to double maxima at 324 and 312 nm. The latter increased in intensity to a maximum at 310 nm by 9 days while there remained a shoulder at 323 nm. No such shifts were observed in similar solutions of roquefortine kept in the dark or on the bench under normal laboratory lighting conditions. Analysis of the concentrated 9-day-old solutions by TLC in solvent system CM confirmed the almost complete conversion of roquefortine to a photoproduct (R_f 0.54) in those solutions exposed to daylight. Preparative TLC afforded material whose mass spectrum indicated that it was an isomer of roquefortine. The

photoproduct was not detected in blue cheese extracts.

Samples of blue cheese of different origin were obtained from local stores and analyzed for the *Penicillium roqueforti* alkaloids (Table I). Analyses were made in duplicate or triplicate in several cases, on radially cut pieces if from the usual round cheese block. Some earlier single analyses included in Table I were made with omission of evaporation of the initial chloroform extract and the second acid extraction, which would result in approximately 8 and 22% lower estimations of roquefortine and isofumigaclavine A, respectively. Three blue cheeses were also trimmed so as to obtain subsamples essentially free of visible mold and also pieces containing as much mold as possible. Results (Table I) reflect the variable distribution of internal mold in individual cheeses and indicate little or no migration of roquefortine into non-moldy parts of the cheese. The detection limit of the method was 0.03–0.05 ppm of roquefortine and about half of this for isofumigaclavine A. Roquefortine was present in all blue cheese varieties examined (Table I); the highest concentration found (6.8 ppm) was in a selected piece with a visually high mold content from the center of a rectangular block of German cheese (actually well separated from the other subsamples of this cheese). Roquefortine and isofumigaclavines A and B were verified in at least one other TLC solvent system, although system CD was not useful for roquefortine in cheese extracts containing tryptamine, which migrated at a similar R_f in this system. Spectroscopic confirmation of identity of roquefortine and isofumigaclavine A was obtained in some cases on the respective compounds isolated from cheese extracts by preparative TLC. The characteristic uv absorption maxima of roquefortine at 240 and 326 nm were verified for five samples, and isofumigaclavine A from three samples showed peaks at 226, 277 (sh), 283, and 293 nm. Mass spectra of roquefortine isolated from four cheese samples showed prominent ions at m/e 389, 320 (base peak), 198, 192, 185, 163, 157, 130, and 108 as observed with the authentic compound (Figure 1a). The mass spectra of isofumigaclavine A isolated from the Finnish and one of the Danish blue cheeses were closely similar to that of standard (Figure 1b) above m/e 105, with prominent ions at m/e 298, 239 (base peak), 223, 197–194, 180, 181, 169–167, 154, 144, and 127. Mass spectroscopic evidence for the presence of isofumigaclavine B (standard spectrum shown in Figure 1c) in the same cheeses was also obtained. Agroclavine (R_f 0.57, system CMN) was not detected in the blue cheese extracts; this was one of three clavine alkaloids suspected by means of paper chromatography to be present in a *Penicillium roqueforti* extract by Taber and Vining (1958).

In view of the very limited toxicological data on roquefortine, an assessment of the possible significance of its consistent presence and the amounts found in blue cheese must await the outcome of further studies. The very small amounts of isofumigaclavine A available have so far precluded any toxicological study. However, the toxicity of the possibly identical roquefortine A (Ohmomo et al., 1975) was reported to be weak; roquefortine A was also stated to be present in various roquefort-type cheeses, together with roquefortine B, although no experimental details were given. Abdel Kader et al. (1969, 1970) have shown that lipids extracted from an Egyptian cheese processed with *Penicillium roqueforti* were toxic to rats, but the toxic factors remain unidentified. In an analogous petroleum ether (bp 30–60 °C) extract of a Danish blue cheese (sample A-2, Table I), we have detected only small amounts of roquefortine (equivalent to less than 0.06 ppm in the cheese) and an amount of isofumigaclavine A equivalent to 0.19 ppm.

LITERATURE CITED

Abdel Kader, M. M., Zaki, A. H., El-Kirdassy, Z. H. M., El-Kammah, B., Bosseila, A. A., *J. Egypt. Med. Assoc.* 52(10), 764 (1969).

Abdel Kader, M. M., Zaki, A. H., El-Kirdassy, Z. H. M., Shoeb, Z. E., Eissa, M. H., *Grasas Aceites (Seville)* 21(4), 197 (1970).

Abe, M., Yamatodani, S., Yamano, T., Kozu, Y., Yamada, S., *Nippon Nogei Kagaku Kaishi* 41(2), 68 (1967).

Bekmakhanova, N. E., *Mikol. Fitopatol.* 8(2), 152 (1974).

Frayssinet, C., Lafarge-Frayssinet, C., Institut de Recherches Scientifiques sur le Cancer, Villejuif, France, personal communication, 1975.

Kanota, K., *Proc. U.S.–Jpn. Conf. Toxic Micro-Org., 1st, 1968*, 129 (1970).

Le Bars, J., Escoula, G., *Aliment. Vie* 62(2), 125 (1974).

Ohmomo, S., Sato, T., Utagawa, T., Abe, M., *Agric. Biol. Chem.* 39(6), 1333 (1975).

Raper, K. B., Thom, C., "A Manual of the Penicillia", Hafner Publishing Co., New York, N.Y., 1968, pp 392–404.

Scott, P. M., Merrien, M.-A., Polonsky, J., *Experientia*, 32(2), 140 (1976).

Shih, C. N., Marth, E. H., *J. Milk Food Technol.* 34, 119 (1971).

Spilsbury, J. F., Wilkinson, S., *J. Chem. Soc.*, 2085 (1961).

Still, P. E., Wei, R.-D., Smalley, E. B., Strong, F. M., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 31(2), 733 (1972).

Taber, W. A., Vining, L. C., *Can. J. Microbiol.* 4, 611 (1958).

Wei, R.-D., Schnoes, H. K., Hart, P. A., Strong, F. M., *Tetrahedron* 31, 109 (1975).

Wei, R.-D., Still, P. E., Smalley, E. B., Schnoes, H. K., Strong, F. M., *Appl. Microbiol.* 25(1), 111 (1973).