Determination of roquefortine C in blue cheese using on-line column-switching liquid chromatography

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Abstract

A method is described for the determination of roquefortine C in (blue) cheese. After liquid–liquid extraction with a mixture of hydrochloric acid and methanol, and filtration, an aliquot is analysed using column-switching reversed-phase liquid chromatography. The recovery of roquefortine C in Fetta cheese is about 85%, the calibration curve is linear from 10 to 2500 ng g^{-1} ($r^2 = 0.998$), and the detection limit is about 10 ng g^{-1}. In different batches of Danish Blue concentrations of 1000–2000 ng g^{-1} of roquefortine C are found. As regards the stability of roquefortine C its half-life in diffuse daylight is ca. 50 min, while after irradiation with ultraviolet light, it is about 10 min. © 1998 Elsevier Science B.V. All rights reserved.

1. Introduction

Penicillium roqueforti is a fungus species which is of interest for agricultural food scientists. It is not found in corn silage, mixed grains, and milled rice [1,2], but it is a source of proteolytic and lipolytic enzymes during maturation of a variety of blue veined cheeses such as Roquefort, Danish Blue, Stilton, Gorgonzola, and Gammelost. *P. roqueforti* is known to produce a number of mycotoxins such as festuclarine [3], roquefortine and isofumigaclarines A and B [4] and PR toxins [5] as secondary metabolises. Although there are, so far,

no reports on toxic effects of these compounds in humans after consumption of fungally-processed cheeses, there is an increasing interest in the production of these toxic metabolises by *Penicillium* species, mainly because there are conflicting reports on the toxicity of roquefortine—also known as roquefortine C—in mice [4,6–8].

Another aspect that has not been studied adequately, is the photostability of roquefortine. For example, Ware et al. [9] determined roquefortine in blue cheese and blue cheese dressings by liquid chromatography (LC) without addressing the photodecomposition of the analyte during analysis. However, Scott and Kennedy [6], who developed a thin-layer chromatographic (TLC) method

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for roquefortine in blue cheese, noticed a significant change in the ultraviolet (UV) absorbance spectra of roquefortine solutions exposed to light. A thorough study on the decomposition of roquefortine under conditions normally used for the determination of this solute, is therefore necessary.

The methods developed by Scott and Kennedy [6] and by Ware et al. [9] both need a laborious and time-consuming sample clean-up resulting in recoveries of < 75%. For routine analysis these methods are obviously not convenient. In this paper a more efficient method is described for the determination of roquefortine in blue cheese at levels as low as 10 ng g^{-1}. After a simple liquid–liquid extraction (LLE) with a mixture of methanol and hydrochloric acid, followed by filtration over filter paper, an aliquot is introduced onto a reversed-phase (RP) column-switching LC system.

2. Materials and methods

2.1. Reagents and solutions

Roquefortine (purity over 98%) was purchased from CSIR, Food Science and Technology (Pretoria, South Africa). Stock solutions of 100 μg ml^{-1} in methanol were prepared freshly every week and stored at 4°C in glass containers completely covered with aluminium foil. Working solutions were prepared daily by diluting the stock solution with the required volume of an appropriate solvent.

Acetonitrile and methanol were of HPLC-grade and were purchased from J.T. Baker (Deventer, The Netherlands). HPLC-grade water was prepared by using a Milli-Q purification system of Millipore (Bedford, MA). All other reagents were of analytical grade and obtained from J.T. Baker.

Fetta, Danish Blue, Gorgonzola and Roquefort cheeses came from a local grocery shop.

The RPLC eluent of acetonitrile—20 mM dipotassium hydrogen phosphate (pH 7.0) (40:60, v/v) was degassed by vacuum ultrasonication (20 min) before use.

The stability experiments were performed by diluting the roquefortine stock solutions to 10 ng ml^{-1} with ethyl acetate, 0.03 M hydrochloric acid or 1 M dipotassium hydrogen phosphate buffer adjusted with 0.03 M hydrochloric acid to pH 7.0. The dilutions were made just before the actual analysis. All solutions were prepared in glass containers and stored in the dark.

2.2. Equipment

The LC system consisted of a Hewlett-Packard (Palo Alto, CA) series 1050 LC pump, a home-made pulse damper (Free University), a 25 cm × 4.6 mm I.D. Vydac (Mojave Hesperia, CA) LC column packed with a 5 μm C-18 bonded silica, a Kratos (Ramsey, NJ) Spectroflow 757 absorbance detector set at 330 nm for the RPLC experiments, or a Hewlett-Packard model 1040 diode-array (DA) detector equipped with a 10 mm flow cell to produce absorbance spectra, and a Hewlett-Packard model 3396A integrator for peak area measurements. LC was performed at ambient temperature using a flow rate of 1.5 ml min^{-1}. Samples were introduced onto the column-switching LC system by means of a Gilson (Villiers-le-Bel, France) model 401 dilutor, equipped with a 1 ml syringe and sample controller.

A home-made 10 × 3.0 mm I.D. precolumn (Free University), packed with a 40 μm C-18 sorbent (J.T. Baker), was used for on-line preconcentration and/or clean-up (Fig. 1). In the preconcentration set-up two six-port switching valves (Valco, Houston, TX) were used, V1 being equipped with a 4.5 ml loop. A Kontron (Zurich, Switzerland) model 414 LC pump was used to wash the precolumn with 4.5 ml of water and to transfer the sample to the precolumn, both at a flow rate of 1 ml min^{-1}.

To determine the breakthrough volume of roquefortine on the C-18 precolumn, the model 401 dilutor was used to pass solutions of the analyte over the precolumn at a flow rate of 1.5 ml min^{-1} for volumes up to 50 ml and 3.0 ml min^{-1} for volumes over 50 ml.

2.3. Stability experiments

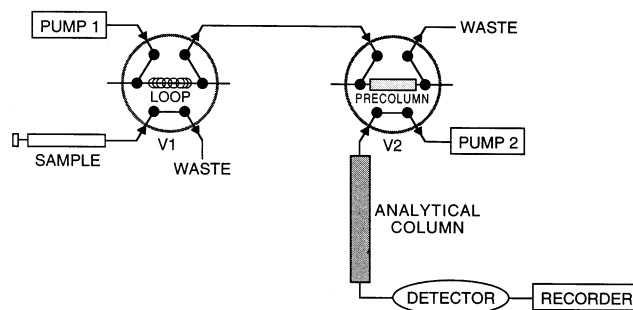During the first series of experiments, the analyte solutions in ethyl acetate or hydrochloric acid

Fig. 1. Instrumental set-up for the preconcentration and LC analysis of roquefortine containing solutions. V1 and V2 are six-port switching valves. The configuration during the LC analysis is given.

were kept in the dark, either at ambient temperature or at 4°C. In a second series of experiments, the solutions were exposed to diffuse daylight or UV radiation produced by a 90 W high-pressure mercury lamp (Philips, Eindhoven, The Netherlands). Exposure to UV radiation was performed in a 10-ml quartz cuvette at a distance of 45 cm from the lamp. The stability experiments, under exposure to diffuse daylight, were performed in transparent glass containers at ambient temperature at a distance of 3 m from the laboratory window.

When ethyl acetate was used as the solvent it was removed before RPLC by transferring 10 ml of the ethyl acetate-containing solutions to a 100-ml round-bottom flask, which was covered with aluminium foil. The flask was connected to a rotary evaporator and the solvent was completely removed under vacuum, at ambient temperature, in ca. 10 min. The residue was dissolved in 10 ml of the RPLC eluent and a 125-μl aliquot was injected.

2.4. Sample clean-up

After grating and mixing of 100–200 g of cheese, a 25-g aliquot was accurately weighed into a 600-ml glass container. Grating was performed with a normal kitchen grater. Thereafter, 150 ml of 0.1 M hydrochloric acid-methanol (70:30, v/v) were added and the mixture was blended for 2–3 min with a medium-speed manual blender. When chloroform or ethyl acetate were used as the extraction solvent instead of hydrochloric acid-

methanol, a Waring blender equipped with a stainless-steel jar was used and the cheese was weighed directly into the jar. After blending, the resulting mixture was filtered over standard filter paper into a 250-ml glass container. Blending and filtration were carried out in the dark.

For samples extracted with the hydrochloric acid-methanol mixture, 20 ml of the filtrate were transferred to a clean glass container, which was covered with aluminium foil. Further clean-up and preconcentration was performed using the 10×3.0 mm I.D. precolumn as mentioned above. If ethyl acetate or chloroform were used, 100 ml of the filtrate were transferred to a 250-ml separatory funnel and 50 ml of a 0.03 M hydrochloric acid solution were added. The organic layer was transferred to another 250-ml separatory funnel and the extraction with hydrochloric acid was repeated. The two aqueous extracts were combined and transferred to another 250-ml separatory funnel, 20 ml of hexane were added and the mixture was gently shaken for 1 min. After phase separation, 20 ml of the aqueous phase were transferred to a glass container which was covered with aluminium foil. Further clean-up was performed as described for the extractions with hydrochloric acid-methanol.

3. Results and discussion

3.1. Preconcentration

In order to increase the sensitivity of the RPLC procedure most samples were preconcentrated us

Table 1
Stability of roquefortine in ethyl acetate after storage in the dark or exposure to diffuse daylight or UV light at ambient temperature

Dark		Daylight		UV light	
Time (h)	Peak[a] area	Time (h)	Peak[a] area	Time (min)	Peak[a] area
0	1	0.0	1	0	1
1	0.95	0.2	0.85	1	0.84
2	0.93	0.5	0.64	5	0.60
6	0.92	1.0	0.58	10	0.46
24	0.91	1.5	0.30	15	0.38
		2.0	0.19	20	0.17
		2.5	0.12	25	0.15
				31	0.14

[a] Peak area at zero time taken as 1.

ing a 10×3.0 mm I.D. precolumn packed with 40 μm C-18 bonded silica (for the set-up see Fig. 1). During preconcentration the 4.5-ml loop of valve V1 was filled with the sample and after switching of V1 its contents were transferred to the precolumn using water as the carrier solvent. Subsequently, the precolumn was washed with 4.5 ml of water after which valve V2 was switched to elute the analyte to the analytical column.

To determine the volume that could be pre-concentrated on the C-18 precolumn, 5–150 ml of 10 ng ml^{-1} solutions of roquefortine in 0.03 M hydrochloric acid were injected into the system. Even after loading of 150 ml of sample no breakthrough was observed. This means that the sensitivity of the method can be improved significantly by a suitable preconcentration rather than a loop-injection procedure.

3.2. Stability of roquefortine-containing solutions

Preliminary experiments clearly showed that roquefortine-containing solutions were not stable when exposed to light [6,10]. In fresh solutions in chloroform, for example, roquefortine had a single absorbance maximum at 337 nm, but after a 24 h exposure to diffused daylight two maxima (312 and 324 nm) showed up. These changes were not found when the solutions were stored in the dark [6]. Analysis of concentrated 9-day-old stock

solutions, which were exposed to diffuse daylight, showed a nearly quantitative photoconversion of roquefortine. Because of this instability a more detailed study was needed.

The determination of roquefortine in blue cheese is frequently carried out after LLE with chloroform or ethyl acetate; to avoid the formation of emulsions, ethyl acetate is nowadays preferred over chloroform [6,9]. The stability studies were performed for solutions in ethyl acetate, 0.03 M hydrochloric acid and 0.03 M hydrochloric acid adjusted to pH 7.0.

3.2.1. Stability in ethyl acetate

After irradiation the ethyl acetate should be removed prior to RPLC. To achieve this several methods were tested. Evaporation of ethyl acetate at ambient or elevated temperature-with or without the use of a stream of dry nitrogen-resulted in a nearly quantitative loss of the analyte. The best method proved to be vacuum distillation at ambient temperature in the dark. Using this procedure no analyte losses occurred.

The solutions of roquefortine which were kept in the dark at ambient temperature or 4°C hardly showed any degradation for at least 24 h. The samples which were exposed to diffuse daylight or UV radiation, however, showed significant degradation (Table 1). From the data a half-life of 50 min can be calculated for exposure to diffuse
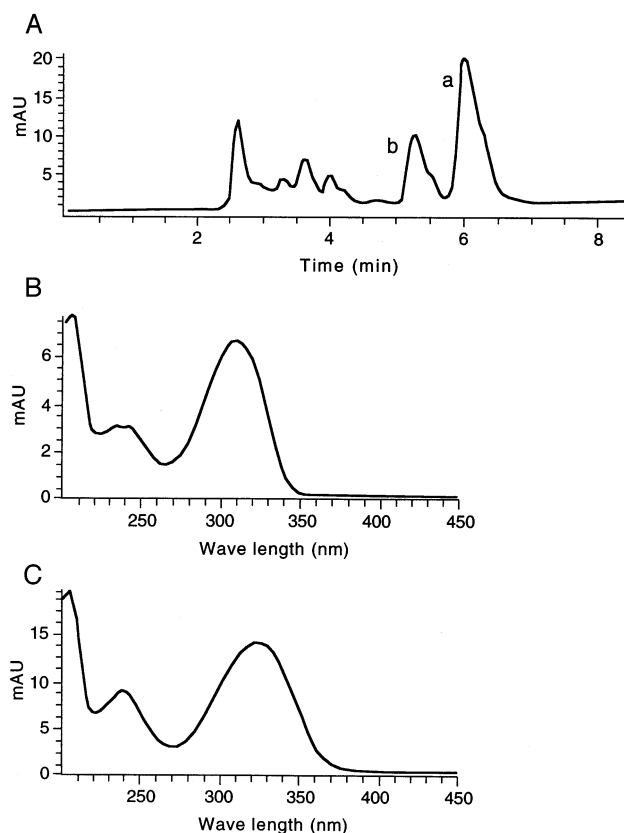
Fig. 2. (A) LC-UV chromatogram of roquefortine-containing solution after photodegradation in ethyl acetate and their UV absorbance spectra (B, C). (a) Roquefortine, (b) degradation product.

daylight, assuming that degradation takes place via first-order kinetics. The half-life of roquefortine decreases to 11 min upon irradiation with UV light.

Degradation of roquefortine in ethyl acetate solutions after exposure to light resulted in at least one ($t_r = 5.3$ min) additional peak in the RPLC chromatogram (Fig. 2A). The UV absorbance spectra of roquefortine itself (Fig. 2B) and the degradation product (Fig. 2C) are rather different. No attempt has been made as yet to elucidate the structure of the degradation product.

3.2.2. Stability in hydrochloric acid and phosphate buffer

Solutions of roquefortine in 0.03 M hydrochloric acid and 0.03 M hydrochloric acid—20 mM dipotassium hydrogen phosphate (pH 7) were also tested for their stability. In one series of experiments the solutions were stored in the dark at ambient temperature; in other series of experiments they were exposed to diffuse daylight or UV light. The conditions were the same as described for the experiments in ethyl acetate.

As expected, the aqueous solutions of roquefortine are relatively stable in the dark. However, when they are exposed to diffuse daylight or UV light they are not stable at all (Tables 2 and 3). A representative chromatogram is given in Fig. 3. In daylight roquefortine seems to have the best stability in a solution of 0.03 M hydrochloric acid. Surprisingly the worst stability is found in ethyl acetate: a solvent that is frequently used for the extraction of roquefortine from cheese [6,9].

Table 2
Stability of roquefortine in 0.03 M hydrochloric acid after storage in the dark or exposure to diffuse daylight or UV light at ambient temperature

Dark		Daylight		UV light	
Time (h)	Peak[a] area	Time (h)	Peak[a] area	Time (min)	Peak[a] area
0.0	1	0.0	1	0.0	1
1.5	0.97	0.5	0.98	5.0	0.57
5.5	0.88	1.0	0.94	10.0	0.43
7.0	0.86	2.0	0.93	15.0	0.37
7.5	0.85	3.5	0.87	20.0	0.35
24.0	0.70	5.0	0.85	25.0	0.32
		6.0	0.80		

[a] Peak area at zero time taken as 1.

Table 3
Stability of roquefortine in 0.03 M hydrochloric acid adjusted to pH 7.0 after storage in the dark or exposure to diffuse daylight or UV light at ambient temperature

Dark		Daylight		UV light	
Time (h)	Peak[a] area	Time (h)	Peak[a] area	Time (min)	Peak[a] area
0.0	1	0.0	1	0	1
1.0	0.95	0.5	0.95	5	0.57
2.0	0.92	1.5	0.77	10	0.36
3.0	0.91	2.5	0.62	15	0.29
4.5	0.90	4.0	0.43	20	0.20
5.5	0.90	5.0	0.34	25	0.11
6.0	0.88	6.5	0.32		
7.0	0.86				

[a] Peak area at zero time taken as 1.

3.3. Sample clean-up

Because roquefortine solutions are not stable in ethyl acetate after exposure to light, the LLE procedures described in the literature can not be safely used [6,9]. Positively, the above experiments show that solutions of the analyte in hydrochloric acid are sufficiently stable to permit sample treatment (0.5–1 h). Besides, at least 150 ml of such a roquefortine solution can be preconcentrated on a C-18 precolumn. An LLE procedure was therefore developed which utilizes a mixture of 0.1 M hydrochloric acid and methanol as the extraction solvent. After blending and grating of ca. 200 g of cheese, a 25 g aliquot was extracted with hydrochloric acid or the hydrochloric acid-methanol mixture (cf. below).

In order to study the repeatability and recovery of the extraction procedure, the resulting filtrate was on-line concentrated using the set-up given in Fig. 1. A 1.0-ml sample was passed through the 10×3.0 mm precolumn at a flow rate of 1.5 ml min^{-1} by means of the model 401 dilutor. The precolumn was washed with 2.0 ml of water at a flow rate of 1.0 ml min^{-1}. The concentrated sample was subsequently transferred online to the analytical column.

Initially the recovery experiments were performed with Fetta cheese and because no roquefortine was found in several batches of this cheese, it was chosen as a blank in all further studies. The actual recovery was determined by spiking 25 g of Fetta cheese with 200 μl of a 206 μg ml^{-1} solution of roquefortine in methanol. The same amount of
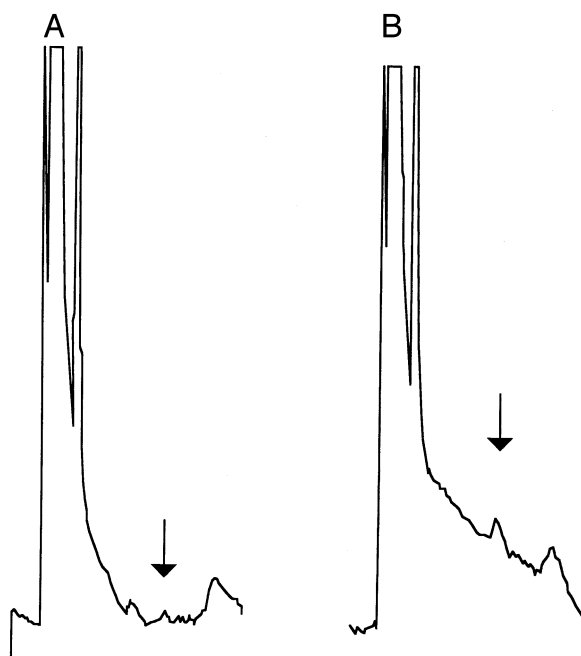
Fig. 3. LC-UV chromatograms of 10 ng ml^{-1} solutions of roquefortine in 0.03 M hydrochloric acid-20 mM dipotassium hydrogen phosphate (pH 7). (A) Before; (B) after exposure to diffuse daylight for 5 h; (C) after exposure for 15 min to UV light.

Fig. 4. LC-UV chromatograms of 25 g of (A) Fetta cheese before and (B) after spiking with 8.24 ng g^{-1} of roquefortine.

roquefortine was added to 150 ml of 0.1 M hydrochloric acid, which was treated in the same way as were the spiked Fetta cheese samples. Using only 150 ml of 0.1 M hydrochloric acid as the extraction solvent the recovery was ca. 60%. To increase the recovery methanol was added to the hydrochloric acid and by using a mixture of 30% of methanol and 70% of 0.1 M hydrochloric acid the recovery increased to ca. 85% (Table 4). Higher

Table 4
Dependence of recovery of roquefortine on percentage of methanol in extraction solvent

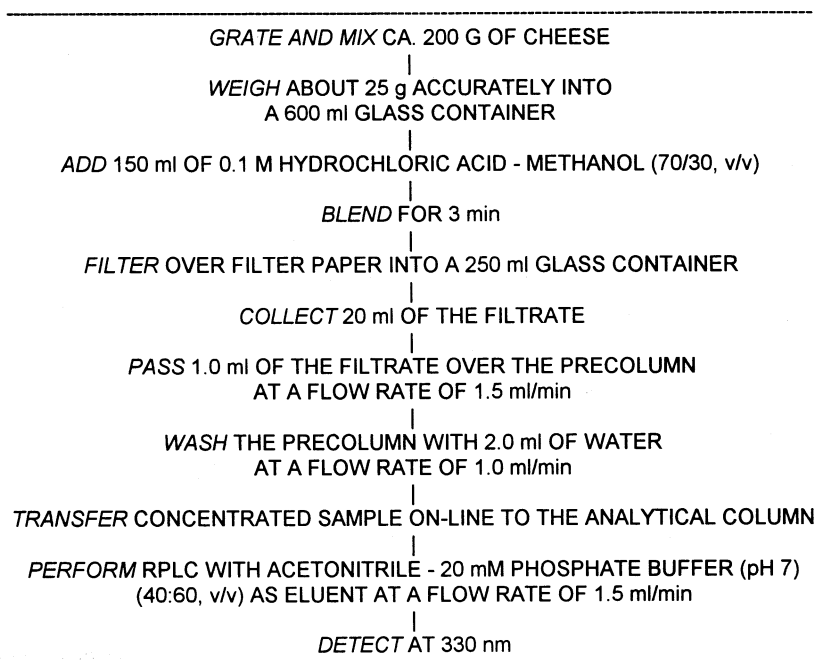| Methanol–0.1 M hydrochloric acid (ml ml^{-1})	Methanol in solvent (%)	Recovery (%)
0–150	0.0	62.6
10–140	6.7	67.3
15–135	10.0	71.5
30–120	20.0	81.7
45–105	30.0	85.6

percentages of methanol could not be used because of breakthrough of roquefortine during the pre-concentration step.

In addition, some extraction experiments were performed with chloroform or ethyl acetate. When using these solvents a blender should be used which does not contain any polymeric material to avoid contamination of the sample. Furthermore, a slightly different procedure was followed using these two extraction solvents (see experimental). The extractions with chloroform and ethyl acetate were not studied in detail since, with ethyl acetate, an emulsion was formed during back extraction with hydrochloric acid, while non-reproducible results were obtained in the case of chloroform.

3.4. Analytical data

The linearity of the whole procedure was tested by spiking Fetta cheese with different concentrations of roquefortine. Portions of 25 g of cheese were spiked with 10, 20, 50 and 100 μl of a 20.6 μg ml^{-1} solution and 50, 100, 150, 200, 250 and 300 μl of a 206 μg l^{-1} solution of roquefortine solution in methanol. The resulting calibration plot ($y = 296.8 \times - 2884.9$) had a regression coefficient, r^2, of 0.998. All samples were analysed in duplicate. Obviously, repeatability is fully satisfactory.

The detection limit of the method (signal-to-

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------------------------------------------------------------------------------------------------
                    GRATE AND MIX CA. 200 G OF CHEESE
                                    |
                    WEIGH ABOUT 25 g ACCURATELY INTO
                        A 600 ml GLASS CONTAINER
                                    |
        ADD 150 ml OF 0.1 M HYDROCHLORIC ACID - METHANOL (70/30, v/v)
                                    |
                            BLEND FOR 3 min
                                    |
        FILTER OVER FILTER PAPER INTO A 250 ml GLASS CONTAINER
                                    |
                    COLLECT 20 ml OF THE FILTRATE
                                    |
            PASS 1.0 ml OF THE FILTRATE OVER THE PRECOLUMN
                    AT A FLOW RATE OF 1.5 ml/min
                                    |
            WASH THE PRECOLUMN WITH 2.0 ml OF WATER
                    AT A FLOW RATE OF 1.0 ml/min
                                    |
    TRANSFER CONCENTRATED SAMPLE ON-LINE TO THE ANALYTICAL COLUMN
                                    |
    PERFORM RPLC WITH ACETONITRILE - 20 mM PHOSPHATE BUFFER (pH 7)
            (40:60, v/v) AS ELUENT AT A FLOW RATE OF 1.5 ml/min
                                    |
                        DETECT AT 330 nm
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* All off-line sample clean-up steps were performed in the dark

Scheme 1.

noise ratio, 3) was ca. 10 ng g^{-1}, as can be seen from Fig. 4 in which a blank and a spiked (8.24 ng g^{-1}) LC chromatogram is shown for Fetta cheese. The detectability can be further improved by transferring a larger fraction (over 1 ml out of 150 ml) of the filtrate over the precolumn. However, detection limits of ca. 10 ng g^{-1} are sufficient to check cheese for the presence of roquefortine.

3.5. Determination of roquefortine in blue cheese

Roquefortine was determined in three types of blue cheese (Danish Blue, Gorgonzola, Roquefort) following the procedure described in Scheme 1. Furthermore, a sample of Gouda cheese, which was suspected to be infected with *Penicillium roqueforti* was analysed. The results are given in Table 5.

As expected rather high concentrations of roquefortine were found in all blue cheeses, but no roquefortine was detected in the Gouda cheese.

4. Conclusions

A straight forward and efficient method is described for the trace-level determination of roquefortine in cheese. The final procedure is given in Scheme 1. Positive features of this procedure are the gain in time, using a combination of a liquid–liquid and a solid phase extraction, the improved sensitivity by using on-line trace enrichment, and the use of solvents (hydrochloric acid-methanol) in which the roquefodine is sufficiently stable. The recovery of roquefortine in Fetta cheese is ∼ 85%, the calibration curve is linear from 10 to 2500 ng g^{-1}, and the detection limit is < 10 ng g^{-1}. In different batches of Danish Blue, roquefortine concentrations > 1000 ng g^{-1} are found. Because of the low stability of roquefortine in the various solvents used in this

Table 5
Determination of roquefortine in cheese and low-fat spread

Cheese	Batch	Roquefortine concentration (ng g^{-1}) ($n = 4$)	RSD (%)
Danish Blue	A1	2190	0.7
	A2	2290	2.0
	B	1170	3.5
Roquefort		705	4.5
Gorgonzola		950	1.2
Gouda		Not detected	

study all sample manipulations are performed and all solutions are stored in the dark.

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