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Determination of sterigmatocystin in cheese by high performance liquid chromatography – tandem masspectrometry

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1
2 1 *Food Additives and Contaminants*

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5 3 **RESEARCH ARTICLE**

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10 5 **Determination of sterigmatocystin in cheese by high performance liquid**
11
12 6 **chromatography – tandem mass spectrometry**

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15 7
16 8 **A. Veršilovskis^{a,b*}, C. Van Peteghem^a, S. De Saeger^a**

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22 13
23 14 **Abstract**

24 15 Different cheese samples, produced in Latvia (8) and Belgium (13), were analysed for their
25 16 sterigmatocystin (STC) content. Only two (9.5 %) of the samples were positive for STC with
26 17 concentration levels of 1.23 and 0.52 $\mu\text{g kg}^{-1}$ respectively. Five (24%) samples contained STC
27 18 above the limit of detection (0.03 $\mu\text{g kg}^{-1}$) but below the limit of quantification (0.1 $\mu\text{g kg}^{-1}$), while
28 19 five samples contained STC above LOD, but below LOQ. A sensitive liquid chromatography –
29 20 electrospray positive ionization – tandem mass spectrometry (LC-MS/MS) method, which was
30 21 previously developed for the analysis of STC in grains, was modified and applied to the analysis of
31 22 STC in cheese. This method involved sample extraction with acetonitrile:water (90:10, v/v),
32 23 defatting with n-hexane, solid phase extraction, separation on a reversed phase C₁₈ column and STC
33 24 detection by LC-MS/MS.

34 25
35 26 **Keywords:** Sterigmatocystin, mycotoxin, cheese, LC-MS/MS, *Aspergillus spp.*, *Aspergillus*
36 27 *versicolor*

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1 Introduction

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6 Mould growth on cheese and, to a less extent, other fermented dairy products stored at low
7 temperatures is a common and recurrent problem for the cheese manufacturer during aging and for
8 the retailer and consumer during storage. Mould growth on cheese causes economic losses from
9 discoloration, poor appearance, and off-flavours. However, of even more serious concern is that
10 some moulds are capable of producing toxic metabolites known as mycotoxins (van Egmond 1989).
11 Mycotoxins are toxic secondary metabolites produced by various fungal species. The presence of
12 these substances in dairy products can have two origins: a) indirect contamination, which results
13 from lactating animals ingesting contaminated feed and b) direct contamination, which occurs
14 because of intentional or accidental growth of moulds on dairy products (van Egmond 1989).
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23 Some of the fungal starters used in cheese manufacturing have been shown to produce various
24 mycotoxins on culture media (Engel and Teuber 1989). Other moulds besides starters may be
25 present in mould cheeses as contaminants, since various fungi are encountered in the environment
26 of the cheese factory and some of these organisms can be potential mycotoxin producers (Lafont et
27 al. 1990; Lund et al. 1995). Reports indicating the occurrence of mycotoxins due to contaminating
28 fungi in mould cheeses are infrequent, in different cheese samples from Finnish supermarkets only
29 mycophenolic acid and roquefortine C were detected in blue cheese samples (Kokkonen et al.
30 2005). The presence of mycotoxins in dairy products is suggested to be related more to
31 environmental factors causing mould growth than to ingestion of mouldy feed by cows (Lafont et
32 al. 1990).
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43 Aflatoxin producers – *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*)
44 were seldom found in cheese. However, *Aspergillus versicolor* (*A. versicolor*), a species known to
45 be capable of producing sterigmatocystin (STC), seemed to be frequently present. *A. versicolor*
46 isolates from cheese and air in the cheese ripening room were all able to produce STC (Lund et al.
47 1995). STC is of interest because of its relationship to aflatoxin. It contains the same 7,8-
48 dihydrofuro[2,3-*b*]furan moiety, which for STC is fused to a hydroxyl- and methoxy- substituted
49 xanthone (Rodricks 1969). STC is a metabolite not only of *A. versicolor*, but also of *A. flavus*, *A.*
50 *Sydowi*, *A. nidulans*, *Bipolaris*, *Chaetomiun* and *Emiricella* spp. (Davis 1981; Terao 1983; Scott
51 1994; Scott 2004; Frisvad and Thrane 2004). Its acute toxicity, mutagenity, cytotoxicity and
52 carcinogenicity have been reviewed by Terao (1983) and it is classified as a 2B carcinogen by the
53 International Agency for Research on Cancer (IARC 1987). The structure of the adduct of STC at
54 the N-guanine of oligodeoxynucleotides was determined (Gopalakrishnan et al. 1992). A 'no
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2 1 significant risk' level for humans of $8 \mu\text{g kg}^{-1}$ of body weight per day was estimated by the State of
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4 2 California (Scudamore et al. 1996). STC has been found in cheese contaminated with *A. versicolor*
5
6 3 (Scott 1989). Lactose, glycerol, high humidity and temperature are stimulating factors in STC
7
8 4 production by *A. versicolor* on cheese (Veringa et al. 1989). Northolt et al. (1980) reported the
9
10 5 occurrence of STC in cheese ripening in warehouses in the Netherlands. However, low
11
12 6 temperatures (5°C to 7°C) should prevent the growth of *A. versicolor* and production of STC. At
13
14 7 these temperatures, the moulds that develop will be *Penicillium* species, which do not produce
15
16 8 aflatoxins or STC (Bullerman 1981).
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18 10 A number of analytical methods have been published to examine STC in cheese matrix. Thin layer
19
20 11 chromatography (TLC) with limits of detection (LODs) around $20 \mu\text{g kg}^{-1}$. UV or postcolumn
21
22 12 fluorescence with aluminium chloride was described for liquid chromatography (LC) detection.
23
24 13 Aluminium chloride was also used as a spray reagent for two dimensional TLC with densitometric
25
26 14 detection (LOD $1 \mu\text{g kg}^{-1}$ in feed) (Domagala et al. 1997). The most sensitive method for STC
27
28 15 detection in cheese was a liquid chromatography – atmospheric pressure chemical ionization – mass
29
30 16 spectrometry (LC-APCI-MS) method with a LOD of $2.4 \mu\text{g kg}^{-1}$ (Scudamore et al. 1996). Therefore
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32 17 no information is available on lower contamination levels which are also of interest.
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34 19 In this study, a previously developed LC – electrospray positive ionization (ESI) – tandem mass
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36 20 spectrometry (MS/MS) method for the determination of STC in different grains (Versilovskis et al.,
37
38 21 2007) (LOD $0.15 \mu\text{g kg}^{-1}$) was modified and applied to the analysis of STC in different cheese
39
40 22 samples. This is the first described LC-ESI-MS/MS method for STC determination in cheese with
41
42 23 solid phase extraction (SPE) and also, the first time cheese samples produced in Latvia and Belgium
43
44 24 were checked for STC contamination.
45 25

46 26 **Materials and Methods**

47 27 *Cheese samples*

48 28 Cheese samples produced in Latvia (8) and Belgium (13) were purchased during first two month of
49
50 29 2008 year from different local supermarkets in Latvia and Belgium. Samples were homogenized by
51
52 30 a food processor (Valentin, SEB, Selongey Cedex, France), and kept frozen (-20°C) before
53
54 31 analysis.
55 32

56 33 *Chemicals and reagents*

57 34 Methanol (HPLC-grade), acetonitrile (HPLC-grade), n-hexane (HPLC-grade) and chloroform
58
59 35 (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Formic acid was from Fluka

(Buchs, Switzerland). Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Brussels, Belgium). Argon (Air Liquide, Belgium) was used as collision gas in mass spectrometry. STC standard was purchased from Sigma (St Louis, MO, USA). A stock solution of $500 \mu\text{g ml}^{-1}$ was prepared by dissolving 5 mg of STC in 10 ml of chloroform. An aliquot (500 μl) of the stock solution was evaporated to dryness under oxygen-free nitrogen at ambient temperature and immediately redissolved in acetonitrile (5 ml) getting a standard solution of $50 \mu\text{g ml}^{-1}$. This was further used to prepare a standard solution of $5 \mu\text{g ml}^{-1}$ of STC in acetonitrile:water (60:40, v/v). This solution was used to spike samples for recovery experiments and to prepare working standards $1.6 \mu\text{g ml}^{-1}$, $0.8 \mu\text{g ml}^{-1}$, $0.2 \mu\text{g ml}^{-1}$, $0.08 \mu\text{g ml}^{-1}$, $0.04 \mu\text{g ml}^{-1}$, $0.008 \mu\text{g ml}^{-1}$ and $0.004 \mu\text{g ml}^{-1}$ as equivalents to $200 \mu\text{g kg}^{-1}$, $100 \mu\text{g kg}^{-1}$, $25 \mu\text{g kg}^{-1}$, $10 \mu\text{g kg}^{-1}$, $5 \mu\text{g kg}^{-1}$, $1 \mu\text{g kg}^{-1}$ and $0.5 \mu\text{g kg}^{-1}$ in cheese matrix.

Sample preparation

The sample preparation was a slight modification of the method of Veršilovskis et al. (2007) for the determination of STC in grains. Ten grams of cheese were weighed and 50 ml of acetonitrile:water (90:10, v/v) and 30 ml of n-hexane were added. The sample was homogenized using a shaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 30 minutes and then centrifuged (3500 rpm, 10 min, + 15°C). The acetonitrile phase was filtered through filter paper 589 (Schleicher & Schuell, Dassel, Germany). Filtered extract (10 ml) was diluted with 30 ml of water and purified using a Strata X (60 mg) SPE column (Phenomenex, Torrance, CA, USA). The SPE column was conditioned with 3.5 ml methanol followed by 3.5 ml of water prior to use. Then, 40 ml of diluted extract was loaded on to the column (using loading reservoirs of 75 ml (UCT, Bristol, PA, UK) and manifold (Waters, Milford, MA, USA)). The column was twice washed with 3.5 ml of acetonitrile:water (30:70, v/v) and STC was eluted with 3 ml of 100 % acetonitrile. The resulting eluate was evaporated to dryness under nitrogen at 37°C and re-dissolved in 250 μl mobile phase (0.02 % formic acid in acetonitrile and 0.02 % formic acid in water (60:40 v/v)). The calibrants were prepared by spiking blank matrix with the standard and prepared in the same way as the samples. Spiked cheese samples were prepared by adding 400 μl , 200 μl , 50 μl , 20 μl and 10 μl of the $5 \mu\text{g ml}^{-1}$ STC standard solution using a digital pipette to 10 g of sample, which was left for 1 h at ambient temperature with occasional agitation to allow the acetonitrile to evaporate. These volumes of standard were equivalent to levels of $200 \mu\text{g kg}^{-1}$, $100 \mu\text{g kg}^{-1}$, $25 \mu\text{g kg}^{-1}$, $10 \mu\text{g kg}^{-1}$ and $5 \mu\text{g kg}^{-1}$ of STC in cheese respectively. Eight replicates at each concentration level were prepared for recovery experiments.

1 *LC-MS/MS analysis*

2 The LC – MS/MS system consisted of a Waters Alliance 2695 separation module with a 100- μ l
3 injection loop (Waters, Milford, MA, USA) coupled to a Quattro LC triple quadrupole mass
4 spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray interface. An
5 electrospray ionization (ESI) probe in the positive mode was used in the analysis of STC. The
6 mobile phase was used in isocratic regime. A Phenomenex Luna C₁₈(2) (5 μ m), 150 x 3.0 mm
7 (Phenomenex, Torrance, CA, USA) column was used. The flow rate was 0.3 ml min⁻¹, column
8 temperature was 30°C and the injection volume was 50 μ l. The parameters of the mass spectrometer
9 were optimized using the STC standard. The best response was recorded with the following
10 parameters: cone voltage 30 V, capillary voltage 3.5 kV, extractor 2 V, radio frequency (RF) lens
11 0.2 V, source temperature 120°C and desolvation temperature 350°C, cone gas flow 108 l h⁻¹,
12 desolvation gas flow 660 l h⁻¹, collision energy 30 eV, dwell 0.2 sec.

13 For the structural identification in selected reaction monitoring (SRM) mode, the molecular ion
14 [M+H]⁺ (m/z = 325) was fragmented within the MS to its product ions (325>310 and 325>281).
15 Argon at a pressure of 3.5 bar was used as collision gas. A calibration curve was constructed using
16 external standardization in matrix. The product ion with m/z = 281 was used for the quantification
17 of STC. The ratio between peaks of STC obtained on two MRM channels (Peak area (325 > 310) /
18 Peak area (325 > 281)) was used for confirmation of the analyte. This ratio should be 0.7 \pm 0.1 for
19 the compound to be confirmed. The MRM chromatograms of blank cheese sample (a), spiked
20 cheese sample (0.5 μ g kg⁻¹) (b), naturally contaminated cheese sample (c) and sample containing
21 traces of STC (d) are shown in **Figure 1**. The following validation parameters were determined:
22 linearity, LOD, limit of quantification (LOQ), recovery and repeatability. The LOD and LOQ of
23 STC were determined by using standards prepared in the blank matrix and calculated using
24 “Masslynx” software integrated calculation (LOD = 3.3 x S/N-ratio; LOQ = 10 x S/N-ratio).

26 **Results and Discussion**

28 *Method performance and validation parameters*

29 In LC-MS methods the food matrix often causes changes in responses because matrix components
30 can interfere with the ionization of the analytes (Tang and Kebarle 1993; Spanjer et al. 2007).
31 Because of this matrix effect, the calibrants were prepared in blank matrix (Gouda cheese produced
32 in Belgium), in two linear ranges from 0.5 μ g kg⁻¹ to 25 μ g kg⁻¹ and from 10 μ g kg⁻¹ to 200 μ g kg⁻¹.
33 The method was linear for STC from 0.5 μ g kg⁻¹ to 25 μ g kg⁻¹ ($y = 2921x + 82$, $R^2 > 0.991$) and
34 from 10 to 200 μ g kg⁻¹ ($y = 2656x + 617$, $R^2 > 0.990$). A tolerance of \pm 10% was accepted for the
35 different calibration points for good linearity. The lowest validated level for STC in cheese was 0.5

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2 1 $\mu\text{g kg}^{-1}$ ($S/N = 25$). The LOD and LOQ were $0.03 \mu\text{g kg}^{-1}$ and $0.1 \mu\text{g kg}^{-1}$, respectively. A good
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4 2 recovery was achieved for STC and the relative standard deviation (RSD, %) demonstrated
5
6 3 acceptable repeatability (**Table 1**).
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9 5 *Presence of STC in cheese samples*

10 6 The results on the presence of STC in cheese are shown in **Table 2**. STC above LOQ was only
11 7 detected in two (15%) of the 13 Belgian cheese samples. In four (50%) of the 8 Latvian cheese
12 8 samples STC was detected above LOD, but below LOQ. Five (24%) of the 21 analysed samples
13 9 contained STC above LOD, but below LOQ and five (24%) samples contained traces of STC. In
14 10 the past only limited studies on the occurrence and stability of STC in cheese were carried out. STC
15 11 was usually detected in Dutch hard cheeses like Gouda or Edam (van Egmond et al. 1982). Northolt
16 12 et al. (1980) found that nine of 39 cheeses were positive for STC. These positive samples were
17 13 obtained from all different cheese categories with levels ranging from 5 to $600 \mu\text{g kg}^{-1}$. In other
18 14 studies the stability of STC in cheese was checked and it was found to be stable for 3 months at
19 15 various temperatures (van Egmond 1986). The occurrence, distribution and stability of STC were
20 16 also studied in Ras cheese in Egypt and 35 % was found positive with a mean concentration of 22
21 17 $\mu\text{g kg}^{-1}$. In Ras cheese contaminated with spores of *A. versicolor*, toxin production started after 45
22 18 days of ripening, reaching a maximum after 90 days. Aged cheese (more than 6 month) inhibited
23 19 toxin production (Abd Alla et al. 1996). Migration studies of STC in Wilstermarsh cheese after
24 20 inoculation with *A. nidulans* and incubation at 15°C or room temperature for 40 days was limited to
25 21 0.5 cm from the cheese surface (Engel and Teuber 1980). Similar results were obtained with six
26 22 naturally contaminated Gouda cheeses on which *A. versicolor* was growing (van Egmond et al.
27 23 1982).
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25 In our study cheese samples were different from soft (like Kamambert (camembert type) and
26 Bouquet Des Moines) to hard (like Parito, Montenegro and Breugel). Most of hard cheeses do not
27 contain any typical moulds producing STC, so contamination can only be possible during cheese
28 making or cheese storage if they are stored at temperatures above 7°C . Also contamination of
29 camembert type cheese is only possible during cheese making and storage, because *Penicillium*
30 *camemberti* does not produce neither spores nor STC. But in one Latvian camembert type cheese
31 sample (Kamambert 1) STC was detected, probably due to contamination during cheese making or
32 during storage. Also other cheese samples contained STC traces or STC above LOD.

34 Our results show that a risk of contamination of cheese with STC exists. These very low
35 concentrations will not acutely affect consumers' health. Only long-term intake of small

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2 1 concentrations of toxins might have possible adverse effects or synergistic effects with other toxins.
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5 3 **Conclusions**

7 4 This is the first simple and sensitive LC-ESI-MS/MS method for the determination of STC in
8
9 5 cheese that can be applied for routine and confirmation analyses. This is the first study of STC in
10
11 6 Latvian and Belgium cheese that indicates a low exposure rate to STC.
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13 7

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25
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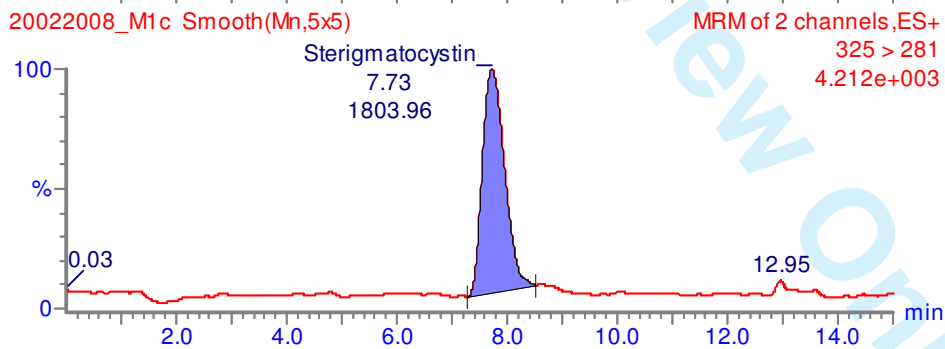
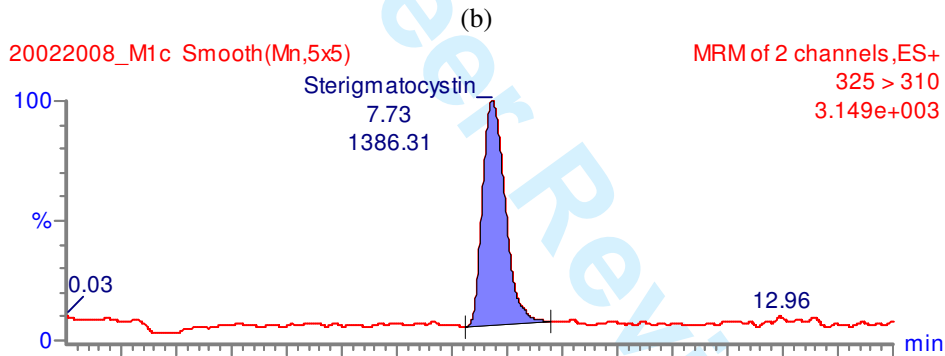
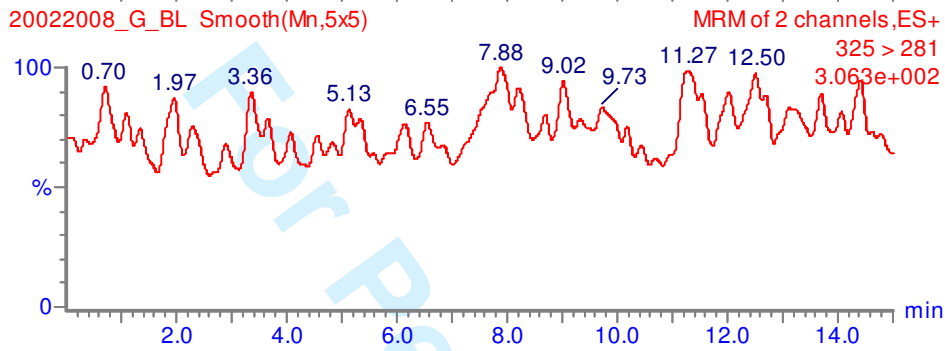
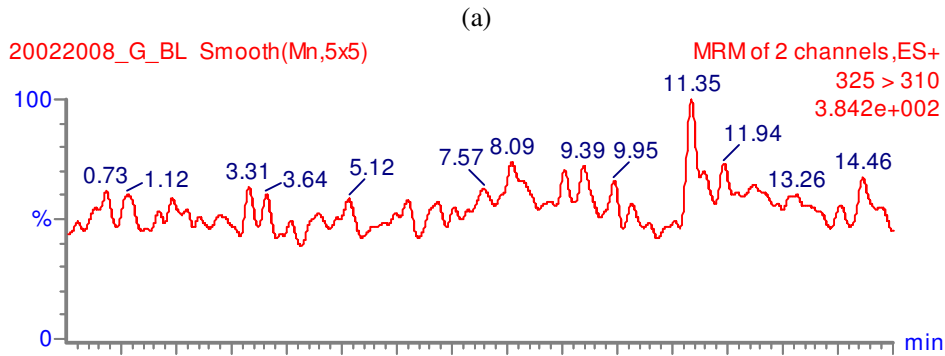
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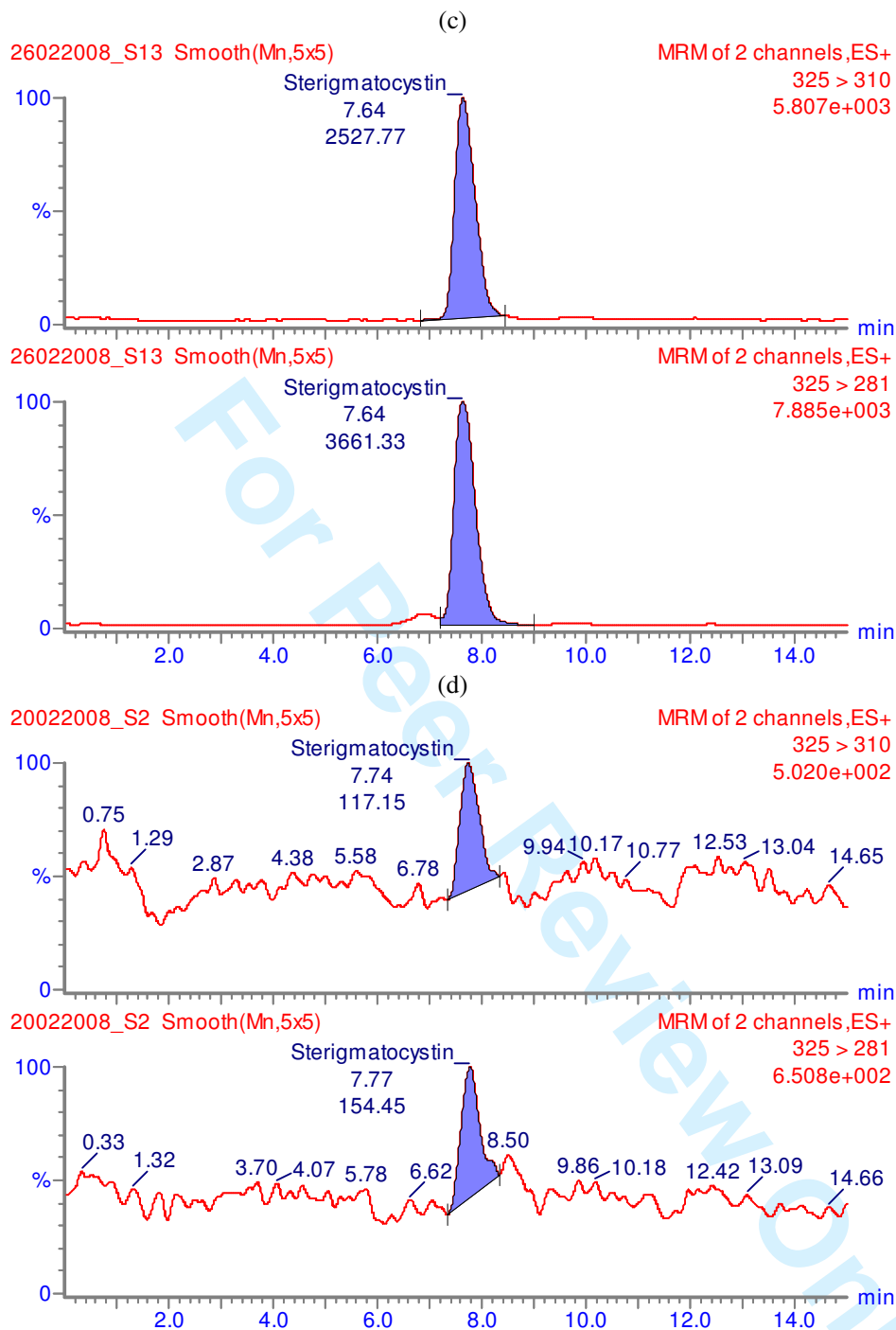


Figure 1. SRM chromatograms of blank cheese sample (a), spiked cheese sample ($0.5 \mu\text{g kg}^{-1}$) (b), naturally contaminated cheese sample (Chevre Affine – contaminated with $1.23 \mu\text{g kg}^{-1}$) (c) and cheese sample containing traces of STC (Krievijas) (d).

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Table 1

Average recovery of STC from spiked cheese samples obtained during two days

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Added concentration, $\mu\text{g kg}^{-1}$	Found concentration (Mean \pm S.D.), $\mu\text{g kg}^{-1}$	Mean recovery (n=8), %	Precision RSD, %
5	5 \pm 1	97	23
10	10 \pm 3	96	27
25	26 \pm 7	103	25
100	104 \pm 25	104	24
200	205 \pm 36	103	17

For Peer Review Only

Table 2

STC presence in different Latvian and Belgian cheese samples

Sample №	Sample Name	Cheese type	Produced in	Found STC concentration, $\mu\text{g kg}^{-1}$	Final decision
1	Zelta	Semi hard	Latvia	0.04	<LOQ
2	Krievijas	Semi hard	Latvia	<LOD	<LOD
3	Hollandes	Hard	Latvia	0.04	<LOQ
4	Parito	Hard	Latvia	0.07	<LOQ
5	Montenegro	Hard	Latvia	<LOD	<LOD
6	Kamambers 1	Soft	Latvia	0.03	<LOQ
7	Kamambers 2	Soft	Latvia	N.D.	<LOD
8	Trikatas Blue	Soft	Latvia	N.D.	<LOD
9	Breugel	Hard	Belgium	<LOD	<LOD
10	Brugs Broodje	Semi soft	Belgium	N.D.	<LOD
11	Brugse Blomme	Soft	Belgium	N.D.	<LOD
12	Bouquet des Moines	Soft	Belgium	N.D.	<LOD
13	Chevre Affine	Soft	Belgium	1.23	Positive
14	Chimay	Semi hard	Belgium	<LOD	<LOD
15	Orval	Semi soft	Belgium	0.03	<LOQ
16	Oud Brugge	Semi hard	Belgium	N.D.	<LOD
17	Passendale	Semi soft	Belgium	N.D.	<LOD
18	St.Paulin	Semi soft	Belgium	<LOD	<LOD
19	Nazareth	Semi hard	Belgium	N.D.	<LOD
20	Watou Special	Soft	Belgium	0.52	Positive
21	Gouda	Hard	Belgium	N.D.	<LOD

LOD = 0.03 $\mu\text{g kg}^{-1}$, LOQ = 0.1 $\mu\text{g kg}^{-1}$, N.D. – not detected

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