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Determination of isopropylthioxanthone (ITX) in milk, yoghurt and fat by HPTLC-FLD, HPTLC-ESI/MS and HPTLC-DART/MS

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Abstract Two new HPTLC methods for quantification of isopropyl-9H-thioxanthen-9-one (ITX) in milk, yoghurt and fat samples have been developed. Extraction of ITX from milk and yoghurt was performed with a mixture of cyclohexane and ethyl acetate by employment of accelerated solvent extraction (ASE). For soy bean oil and margarine, a simple partitioning of ITX into acetonitrile was used. ITX and 2,4-diethyl-9H-thioxanthen-9-one (DTX) used as internal standard have been separated on silica gel 60 HPTLC plates with a mixture of toluene and *n*-hexane (4:1, v/v) and on RP18 HPTLC plates with a mixture of acetonitrile and water (9:1, v/v). Development was performed anti-parallel from both plate sides leading to a throughput of 36 separations in 7 min. Fluorescence measurement at 254/400 nm was used for quantification. Limits of detection (S/N of 3) have been established to be 64 pg for ITX and DTX on both types of HPTLC plates. In fatty matrix (spiked butter) LOD of ITX was determined to be 1 $\mu\text{g kg}^{-1}$. In the working range monitored (20–200 $\mu\text{g kg}^{-1}$) polynomial regression of ITX showed a relative standard deviation (sdv) of $\pm 1.51\%$ ($r=0.99981$). Starting with the limit of quantification the response was linear (sdv= $\pm 2.18\%$, $r=0.99893$). Regarding repeatability ($n=9$) a coefficient of variation (CV) of 1.1 % was obtained for ITX at 32 ng on silica gel plates and of 2.9 % on reversed-phase plates. Repeatabilities ($n=4$) of ITX determination at 20, 50 and 100 $\mu\text{g kg}^{-1}$ in milk, yoghurt, soybean oil and margarine showed CVs between ± 1.0 and 6.4 %. The results prove that modern planar chromatography is a rapid and cost-efficient alternative method to quantify ITX in milk-based or fatty matrices. Only positive results are confirmed by online ESI/MS in the SIM mode (LOQ 128 pg) and by DART/MS involving a minimal employment of the MS device, which is a further advantage

of HPTLC. Overall mean recovery rates of ITX at 20 or 50 and 100 $\mu\text{g kg}^{-1}$ ($n=8$) were 41 % for milk, 70 % for yoghurt, 6 % for margarine and 12 % for soy bean oil. However, with the internal standard correction recoveries were about 130 % for milk and yoghurt and 70 and 97 % for margarine and soy bean oil, respectively.

Keywords Planar chromatography · Isopropyl-9H-thioxanthen-9-one (ITX) · 2,4-Diethyl-9H-thioxanthen-9-one (DTX) · HPTLC-ESI/MS · HPTLC-DART/MS

Introduction

Isopropylthioxanthone (ITX) is used as photoinitiator in UV inks applied to paper- or plastic-based packaging materials. ITX has recently been found in a number of foods such as ready-to-feed infant formula. Over 30 million litres of milk were retracted by producers in Italy, France, Spain and Portugal in November 2005. Various sources of food contamination have been discussed such as migration of ITX into the food from preformed packaging materials or copying by rolling of the packaging material which enables the contact of outside with inside. Inks applied to food packaging materials are not covered by specific European legislation. However, according to Regulation (EC) No. 1935/2004 materials and articles in contact with food shall be manufactured in such a way that they do not “transfer their constituents to food in quantities which could endanger human health, bring about an unacceptable change in the composition of the food or bring about deterioration in the organoleptic characteristics thereof”.

The composition of the food itself obviously influences the ITX levels found at present up to some hundred $\mu\text{g kg}^{-1}$, e.g. fat-containing foods are more affected than water-based products. Based on the levels reported the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) of the European Food

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Safety Authority (EFSA) advises that the presence of ITX in foods, whilst undesirable, does not give cause for health concern based on the assessment of possible exposure to this substance and its genotoxic potential [1]. Unfortunately, there are no data available at present on aspects other than genotoxicity. Furthermore, it is not only the monitoring of ITX that is of concern: discussions about other photoinitiators like 2-ethylhexyl-4-dimethylamino-benzoate (EHDAB), 4,4'-bis(diethylamino)-benzophenone and 4,4'-bis(dimethylamino)-benzophenone are also underway.

In light of this background, a rapid analytical screening method was required to investigate the absence of ITX in infant formula, milk and milk-based products, soy bean beverages, fruit juices, fruit nectars and other drinks and foods, like cacao powder or olive oils, packed in the same manner. So far only a few suitable analytical methods have been reported, these being GC-MS for determination of ITX in milk up to an LOD of $10 \mu\text{g kg}^{-1}$ [Eurofins (2006, private communication)], HPLC-MS for determination in various milk products and margarines up to the lower $\mu\text{g kg}^{-1}$ range [2] and HPLC-FLD up to $10 \mu\text{g kg}^{-1}$ restricted by confirmation via DAD up to $50 \mu\text{g kg}^{-1}$ [Rothenbacher T (2005, private communication)]. However, in response to the high sample throughput required, the development of an HPTLC-MS method was our primary concern.

Experimental

Materials

All solvents used for sample preparation or chromatography were of gradient grade or distilled prior to use. For mass spectrometric detection MS-grade solvents were used. 2,4-Diethyl-9H-thioxanthen-9-one used as internal standard (Sigma-Aldrich, Steinheim, Germany, Fig. 1a) and isopropyl-9H-thioxanthen-9-one (mixture of 2- and 4-isomers, Sigma-Aldrich, Steinheim, Germany, Fig. 1b) were of 98 % and 97 % purity, respectively.

Hydromatrix (Bulk Isolute HM-N sorbent) used for ASE was purchased from Separtis GmbH (Grenzach-Wyhlen, Germany).

For stability checks, ITX was dissolved ($0.2 \mu\text{g mL}^{-1}$) in acetonitrile and methanol, both containing 0.1 % formic acid, and stored at 5°C ; $100\text{-}\mu\text{L}$ aliquots were analysed on silica gel plates at defined time intervals.

For standard application during method development, solutions of ITX ($0.32 \mu\text{g mL}^{-1}$) and DTX ($1 \mu\text{g mL}^{-1}$),

both dissolved in acetonitrile, were used. For constructing the calibration curve, a ten-fold concentrated standard solution has been applied. Detection limits were determined with ITX and DTX solutions of 32 ng mL^{-1} . Solutions for initial MS spectra recording were 0.1 %.

Silica gel 60 (article no. 1.05641) HPTLC plates and RP18 HPTLC plates (article no. 1.05914), both $20 \times 10 \text{ cm}$ (Merck, Darmstadt, Germany) were employed as stationary phases.

Sample preparation

Milk-based matrix

For milk-based samples, the accelerated solvent extractor ASE-200 (Dionex, Idstein, Germany) was used. A representative aliquot of the sample, i.e. 4 mL milk or 4 g yoghurt, was weighed into a 50-mL beaker, $25 \mu\text{L}$ DTX solution ($8.0 \mu\text{g mL}^{-1}$ acetonitrile) was added as internal standard, and the mixture was thoroughly mixed with the sample by means of a spatula. In addition, blank samples were spiked with 40 or 200 μL of an ITX solution ($2.0 \mu\text{g mL}^{-1}$ acetonitrile) resulting in levels of 20 and $100 \mu\text{g kg}^{-1}$, respectively. The sample was then mixed with 4 g hydromatrix and transferred to a 22-mL ASE extraction cell, which was finally filled up with hydromatrix. The optimized ASE settings were as follows: solvent cyclohexane/ethyl acetate (1:1, v/v), pressure 14 MPa, temperature 100°C , preheat 0 min, heat 5 min, static 5 min, flush 60 %, purge 140 s (nitrogen), cycles 2. Total extraction time was about 20 min and the final extract volume about 35 mL. The extract was dried over 4 g sodium sulfate, filtrated into a 50-mL centrifuge vessel and evaporated to dryness by using a centrifuge evaporator (Centrivap Cold Trap, Labconco, Kansas City, USA) at 45°C and 0.2 mbar (ca. 60 min). The residue was taken up in 500 μL ethyl acetate and transferred into a 2-mL sampler vial, whereafter the centrifuge vessel was rinsed twice with 500 μL ethyl acetate. The combined extract was evaporated to dryness under a gentle stream of nitrogen on a heating plate (50°C). To the residue obtained 250 μL acetonitrile was added. ITX or DTX was extracted from the matrix by partitioning at 50°C (oven) for 30 min in a shaker operating at 600 min^{-1} (KS 125 basic, IKA Labortechnik, Staufen). Finally the vial was placed into a refrigerator (5°C) to crystallize undissolved matrix compounds. The clear acetonitrile layer was transferred into a 300- μL sampler vial and subjected to HPTLC analysis.

Margarine and soy bean oil

A representative aliquot of 1 g margarine or soy bean oil was weighed into a 4-mL screw cap vial and $25 \mu\text{L}$ of a DTX solution ($8.0 \mu\text{g mL}^{-1}$ acetonitrile) and 1 mL acetonitrile were added. For the margarine sample, 200 mg magnesium sulfate was also added. Extraction was performed by partitioning at 50°C (oven) for 30 min in

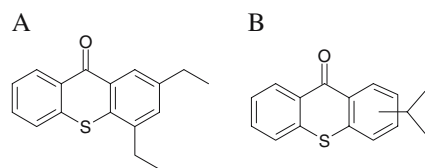


Fig. 1 Structure formulae of **a** 2,4-diethyl-9H-thioxanthen-9-one (DTX) and **b** isopropyl-9H-thioxanthen-9-one (ITX)

a shaker operating at 600 min^{-1} . Thereafter the vial was placed into a refrigerator ($5 \text{ }^\circ\text{C}$) for phase separation. The clear upper acetonitrile layer was transferred into a sampler vial and subjected to HPTLC analysis.

Migration experiment

To simulate a migration process, $200 \text{ }\mu\text{L}$ of an ITX solution ($100 \text{ }\mu\text{g mL}^{-1}$ acetonitrile) was pipetted on a round filter paper (O 5 cm), which was air-dried and placed into a 100-mL glass bottle. UHT milk (100 mL) was added and the bottle was stored in a refrigerator ($5 \text{ }^\circ\text{C}$) for 2 weeks. Sample extraction followed the procedure described in "Milk-based matrix".

HPTLC methods

Stationary phases comprised silica gel 60 or RP18 HPTLC plates, cut to smaller sizes (TLC Plate Cutter, Sarstedt, Nümbrecht, Germany) if necessary. For mobile phase optimization the HPTLC Vario Chamber (CAMAG, Muttenz, Switzerland) was used. Therefore standard solutions were applied as a 95-mm band by an Automatic TLC Sampler 4 (ATS4, CAMAG). After application seven lines were scraped off the plate (HPTLC Scoring unit, CAMAG) resulting in 6 tracks for mobile phase testing. For quantitative determination, standard solutions were sprayed as 7-mm bands by an Automatic TLC sampler 4 (ATS 4, CAMAG). Tracks of 9-mm-distance were applied to the plates, a distance of 23 mm from both sides and 8 mm from the lower edge resulting in 18 tracks per plate side. For determination of the limits of detection 2–12 μL of standard solutions (DTX and ITX 32 ng mL^{-1} each) have been applied. For quantification, application volumes of $30 \text{ }\mu\text{L}$ were used for samples and 3–30 μL (9.6–96 ng) of an ITX solution ($3.2 \text{ }\mu\text{g mL}^{-1}$) covering the working range of 20–200 $\mu\text{g kg}^{-1}$, i.e. for a 4-g milk sample. The ITX standard starting zone was oversprayed with $3 \text{ }\mu\text{L}$ (24 ng) of the internal standard DTX ($8.0 \text{ }\mu\text{g mL}^{-1}$). After drying the starting zones for 1 min, silica gel plates were developed with a mixture of toluene and *n*-hexane (4:1, v/v) and reversed-phase plates with a mixture of acetonitrile and water (9:1, v/v) both up to a migration distance of 50 mm in the Horizontal Developing Chamber (HDC 20 \times 10 cm, CAMAG). Bilateral development at RT and 22 % relative air humidity lasts 7 min for simultaneous separation of 36 chromatographic runs. After drying for 1 min, densitometry was performed via fluorescence measurement at UV 254/ $>$ 400 nm with a TLC Scanner 3 (CAMAG); evaluation was performed via peak height. For digital documentation the DigiStore 2 Documentation System (CAMAG) consisting of illuminator Reprostar 3 with digital camera Baumer optronic DXA252 was applied in the reflectance mode at UV 254/ $>$ 400 nm. A 2-s exposure time and gain of 1 were chosen. Data obtained were processed with winCATS software, version 1.4.1 (CAMAG).

Confirmation by ESI/MS

After scanning the plate, the exact positions of ITX were marked with a pencil. To extract the analytes from the plate a mixture of methanol and ammonium formate buffer (10 mM, pH 4) 95:5 (v/v) was used as elution solvent. The flow of 0.1 mL min^{-1} was provided by an HPLC pump HP 1100 (Agilent Technologies, Palo Alto, USA). The analytes were extracted from the plate by using a slightly modified ChromeXtraktor (ChromAn, Holzhausen, Germany) [Alpmann A and Morlock D (2006, in preparation)]. Mass spectrometric measurement was performed with VG Platform II Quadrupole electrospray mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The capillary voltage was set to 3.5 kV and the cone voltage to 30 V. The pressure of the drying gas was adjusted to 250 bar and the nebulizing gas to 8 bar. SIM and full scan measurements were carried out in ESI⁺ mode. For data acquisition and processing Mass Lynx 3.2 software was used.

Confirmation by DART/MS

After scanning the plate, the exact positions of ITX were marked with a pencil. The HPTLC plate was cut with the TLC Plate Cutter (Sarstedt) and the zones of interest were directed into the charged gas stream of DART which was installed on an AccuTOF CS (Jeol (Europe) SA, Croissy sur Seine, France). Helium was used as gas with a flow rate of 1 L min^{-1} and the gas heater was set to $275 \text{ }^\circ\text{C}$. The orifice distance was fixed to 15 mm. The needle potential was 2.5 kV. For positive-ion detection the potentials of the second perforated electrode and the grid electrode were biased to positive potentials of 100 V and 250 V, respectively.

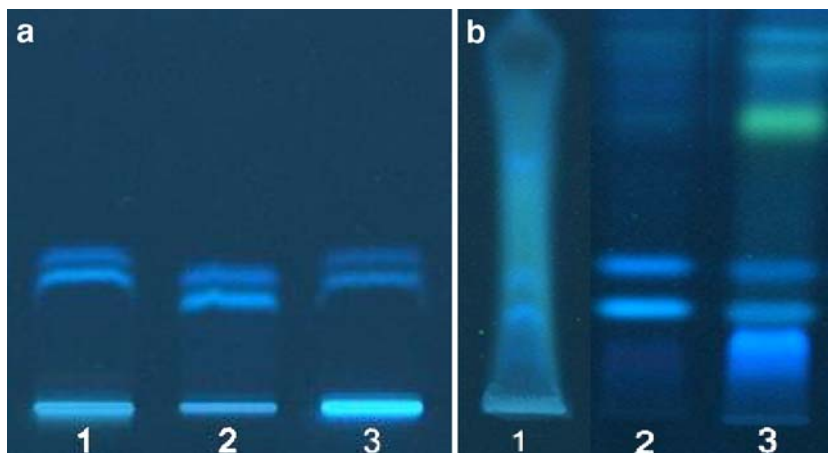
Results

Sample preparation

Accelerated solvent extraction (ASE) has become an important extraction technique in contaminant and residue analysis of food, feeding stuff and environmental matrices [3–5]. To the best of our knowledge, ASE has not been applied for extraction of ITX from food so far. Thus applicability of ASE was investigated for the extraction of ITX from milk-based matrix, because ASE allows short extraction times, low solvent use, increased extraction yields, provides a high level of automation, and thus streamlines analysis. Various parameters were tested to optimize extraction conditions. Of major concern was the selective extraction of ITX from the milk-based matrix.

Different solvents for extraction have been investigated, i.e. acetonitrile, ethyl acetate and a mixture of cyclohexane and ethyl acetate (1:1, v/v). Extractions with acetonitrile or ethyl acetate led to respective higher dry extract masses after evaporation, and thus caused matrix interferences to a

Fig. 2 Track images showing the influence of different ASE extraction solvents (1–3) on chromatography due to co-extracting matrix of milk: **a** silica gel 60 HPTLC plate and **b** RP18 HPTLC plate: 1 acetonitrile, 2 cyclohexane/ethyl acetate (1:1, v/v) and 3 ethyl acetate



higher degree (Fig. 2). Strong matrix interferences could be observed as a chromatographic barrier during the run leading to a disturbed zone shape, e.g. acetonitrile extract on RP phases (Fig. 2b, track no. 1), whereas weaker ones generated more or less hR_F value shifts. Due to the converse chromatographic separation mechanism on RP18 phases, partly or even unacceptable fluorescent track backgrounds could be noticed for extractions with ethyl acetate and acetonitrile, respectively (on silica gel phases these fluorescent matrix interferences are clearly fixed at the starting zone). For ASE extraction the solvent mixture of cyclohexane and ethyl acetate (1:1, v/v) was the best compromise between yield and co-extracting matrix interferences on both plate types. When acidic samples like milk or yoghurt were neutralized with sodium hydrogen carbonate (addition of 100 mg to 4 g sample) before extraction respective matrix interferences could be reduced, indicating the presence of partly acidic co-extracted matrix interferences in the previous case. However, ITX yield was also reduced and consequently this approach was not of major concern.

To further reduce matrix loads of the final solutions, different solvents were tested for selectively taking up the extract residues. In a model setup, samples of 100 mg butter fat were spiked with 80 ng ITX and 200 ng DTX and taken up in 250 μ L toluene, acetonitrile and methanol.

Aliquots (30 μ L) were applied onto the silica gel plate. In the case of toluene ITX and DTX, zones were distorted due to the complete dissolution of fat and thus could not be evaluated quantitatively. Better results were obtained by the use of acetonitrile or methanol, which only slightly dissolve the fatty matrix. Acetonitrile gave the best compromise between yield and matrix interferences (Table 1). Co-extraction of the fatty matrix could easily be seen on the planar chromatogram by white-light illumination in the reflectance and transmission mode of the DigiStore 2 device. The quantity of co-extracted matrix influences the chromatographic position on the plate and could be expressed by the shift of the migration distance (MD) or hR_F value. Adding water to the solvents led to minor shifts due to less extracted fatty matrix; however, this also resulted in unacceptable yields.

The advantage of using acetonitrile or methanol for taking up the dry extract residue is that the small solvent volume (250 μ L) does not dissolve matrix components completely leading to a further selective extraction step of ITX. The partitioning of ITX from a milk extract into methanol was investigated after periods of 1, 2, 4, 8 and 18 h of equilibration and it could be ascertained that after 1 h the partitioning was already in equilibrium.

The stability of ITX under acidic conditions could come into question if curdled milk products are extracted.

Table 1 HPTLC results of butter fat samples (100 mg) spiked with ITX (80 ng) and DTX (200 ng), and extracted with different solvents; application volume 30 μ L each, maximally (100 %; standard solution as reference) resulting in 9.6 ng ITX and 24 ng DTX absolutely on the plate

Solvent	MD shift (mm) due to matrix		hR_F shift due to matrix		Yield	
	MD _{DTX}	MD _{ITX}	hR_{FDTX}	hR_{FITX}	DTX (%)	ITX (%)
Standard solution	14.1	16.0	15	19	100	100
Toluene	(18–22)	(18–22)	n.d.	n.d.	n.d.	n.d.
Acetonitrile	17.8	19.2	23	27	44	45
Methanol	17.7	19.2	23	27	34	35
Methanol/water (9:1,v/v)	14.9	16.8	16	21	13	17
Methanol/water (8:1,v/v)	14.7	16.6	16	20	7	10

n.d. (not determined) due to heavy matrix: ITX and DTX were distorted and shifted to one wavy band
MD migration distance

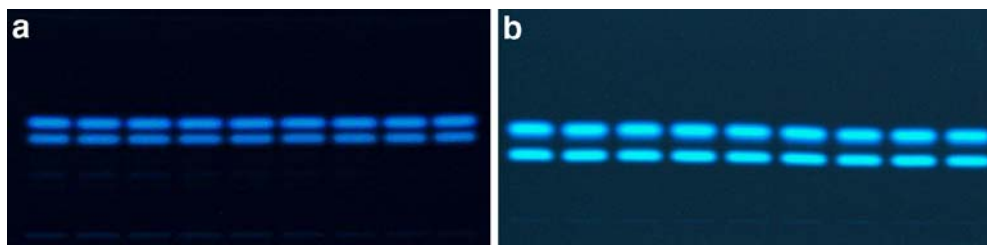


Fig. 3 Repeatabilities ($n=9$) of the planar chromatographic determination via fluorescence measurements at UV 254/>400 nm by illumination (reflectance) at 254/>400 nm: **a** silica gel 60 HPTLC plate, toluene/*n*-hexane (3:1, v/v), coefficient of variation was $\pm 1.1\%$

for both DTX (24 ng, hR_F 20) and ITX (32 ng, hR_F 26). **b** RP18 HPTLC plate, acetonitrile/water (9:1, v/v), coefficient of variation was $\pm 2.9\%$ for both DTX (32 ng, hR_F 25) and ITX (32 ng, hR_F 37)

However, acidified standard solutions both in methanol and acetonitrile were stable for 7 days, as checked by HPTLC. Thus more acidic sample extracts taken up in acetonitrile were also proved to be stable for at least 7 days.

Planar chromatography

So far no planar chromatographic method exists for analysis of ITX. First, a suitable selectivity had to be found for separation of ITX from matrix and the internal standard DTX. ITX and DTX (Fig. 1) differ only in the thioxanthone substitution, i.e. the empirical formulae differ by just one methylene group. Our primary approach was focussed on silica gel plates due to cost-effectiveness. For this purpose the HPTLC Vario Chamber was employed in which 6 solvents can be tested on a channelled plate sized 10×10 cm with only 1 mL mobile phase each. The best solvent selectivity was chosen according to the cost-effective and time-saving guidelines given elsewhere [6]. For the first two levels (testing pure and solvent-strength-adapted solvents) only one plate of 10×10 cm was necessary. The solvents tested, i.e. *tert*-butyl methyl ether, diisopropyl ether, ethanol, butanol, dichloromethane and toluene, were selected from different selectivity groups according to Snyder [7] and 2- to 3-fold diluted with *n*-hexane to adapt solvent strength. Toluene was found to show a satisfying selectivity. This mobile phase was transferred to the chamber (HDC) intended for routine use and chromatography was stopped at a migration distance of 50 mm (developing time 7 min) to enable simultaneous development from both plate sides.

Resolution was slightly better on HPTLC plates than on TLC plates leading to a final resolution of 1.0 which was sufficiently for quantification (4σ separation). DTX showed a hR_F value of 20 and ITX had a hR_F value of 26 (Fig. 3a). To increase the hR_F values slightly a mixture of toluene and *n*-hexane (4:1, v/v) was preferred later on. Regarding repeatability ($n=9$) the coefficients of variation of DTX at 24 ng and ITX at 32 ng were established to be both $\pm 1.1\%$.

Resolution was best on RP18 HPTLC plates developed with a mixture of acetonitrile and water (9:1, v/v) to a migration distance of 50 mm (developing time 7 min). A 6σ separation, i.e. a resolution of 1.5, was obtained. DTX showed a hR_F value of 25 and ITX a hR_F value of 37 (Fig. 3b). Repeatability ($n=9$) was satisfactory, i.e. the coefficients of variation for DTX and ITX both at 32 ng were established to be $\pm 2.9\%$ each.

On RP18 phases UV-absorbing matrix interferences almost coeluting with DTX could be observed, which made evaluation via an internal standard difficult due to fluorescence quenching effects. However, ITX evaluation via recovery rates was still possible. Owing to increased robustness regarding matrix interferences, we focussed on internal standard evaluation on silica gel plates. Repeatabilities of the ITX determination over the whole procedure were given in Table 2. The coefficients of variation of ITX extracted from milk, yoghurt, margarine and soy bean oil were between $\pm 1.0\%$ and $\pm 6.4\%$ spiked at different concentration levels, i.e. $20 \mu\text{g kg}^{-1}$, $50 \mu\text{g kg}^{-1}$ and $100 \mu\text{g kg}^{-1}$. Depending on the type of matrix, overall mean recoveries calculated by the internal standard method ranged between 70 and 130 % (Table 2).

Table 2 Repeatabilities (CV) and recovery rates of the ITX determination (internal standard method) in milk, yoghurt, margarine and soy bean oil on HPTLC plates silica gel 60

	Milk ($n=4$)		Yoghurt ($n=4$)		Margarine ($n=4$)		Soy bean oil ($n=3$)	
Target value ($\mu\text{g kg}^{-1}$)	20	100	20	100	50	100	50	100
\bar{x} ($\mu\text{g kg}^{-1}$)	26	132	24	136	37	66	46	102
CV (%)	2.8	5.7	1.6	4.9	6.4	4.1	1.5	1.0
Recovery (%)	130	132	120	136	74	66	92	102
Correction factor f_c	0.77	0.76	0.83	0.74	1.34	1.51	1.09	1.01
Overall recovery (%)		130		70		97		
Overall f_c		0.78		1.43		1.05		

Table 3 Absolute recovery rates of ITX determined at 20 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ in milk and yoghurt, and at 50 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ in margarine and soy bean oil; for DTX respective recovery rates were determined at 50 $\mu\text{g kg}^{-1}$

ITX	Milk ($n=4$)		Yoghurt ($n=4$)		Margarine ($n=4$)		Soy bean oil ($n=3$)	
Target value ($\mu\text{g kg}^{-1}$)	20	100	20	100	50	100	50	100
Mean Rec _{ITX} \bar{x} (%)	37	45	63	76	5	6	11	13
CV (%)	11.1	16.2	5.9	6.3	13.8	5.3	3.4	7.4
Overall Rec _{ITX} \bar{x} (%)	41		70		6		12	
DTX	Milk ($n=8$)		Yoghurt ($n=8$)		Margarine ($n=8$)		Soy bean oil ($n=6$)	
Target value ($\mu\text{g kg}^{-1}$)	50		50		50		50	
Mean Rec _{ITX} \bar{x} (%)	38		59		12		13	
CV (%)	15.1		6.7		3.7		5.2	

Absolute mean recovery rates of ITX at 20 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ were determined to be 41 % for milk and 70 % for yoghurt; respective values at 50 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ were 6 % for margarine and 12 % for soy bean oil (Table 3). For DTX at 50 $\mu\text{g kg}^{-1}$ mean recovery rates ($n=8$) were established to be 38 % for milk, 59 % for yoghurt, 12 % for margarine and 13 % for soy bean oil. DTX was less extractable than ITX from milk and milk products; the converse was observed for margarine. Both substances were equally extractable from the pure oil matrix. Therefore different correction factors (f_c , Table 2) can be taken into account to calculate the more ‘true’ value.

Scanning the tracks with excitation at 254 nm instead of 365 nm or 313 nm (which are other major lines of the mercury vapour lamp) improved the signal by factors of 2.3 for both substances (254 nm instead of 365 nm) and of 1.4 and 3.0 for DTX and ITX, respectively (254 nm instead of

313 nm). The optimal irradiation wavelength of 254 nm and the factors calculated were confirmed by the fluorescence excitation spectra of ITX and DTX (Fig. 4).

Analytical response established over a wide range (1:100) between 1.2 and 120 ng was fitted best using a polynomial regression ($y = -0.005x^2 + 4.645x - 6.518$), and a relative standard deviation of the calibration function of ± 1.26 % (coefficient of correlation 0.99995) was obtained (not shown). In the working range monitored between 20–200 $\mu\text{g kg}^{-1}$, the analytical response of ITX resulted in a relative standard deviation of the calibration function of ± 1.51 % (coefficient of correlation of 0.99981, data not shown).

In a narrower or lower calibration range, the analytical response was linear. Starting at the limit of determination, even in this very low range of 128 pg to 640 pg the functional correlation on silica gel plates was very good, i.e.

Fig. 4 Fluorescence excitation spectra (200–400 nm) of ITX (pale-grey spectrum, 5 ng) and DTX (dark-grey spectrum, 14 ng) on a silica gel plate confirming the optimal irradiation wavelength of 254 nm for

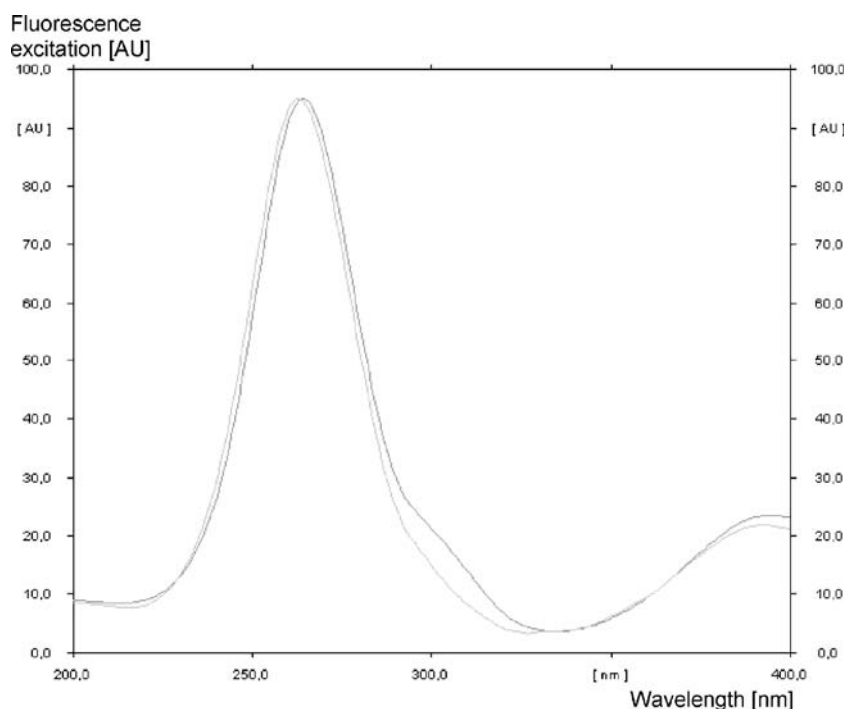
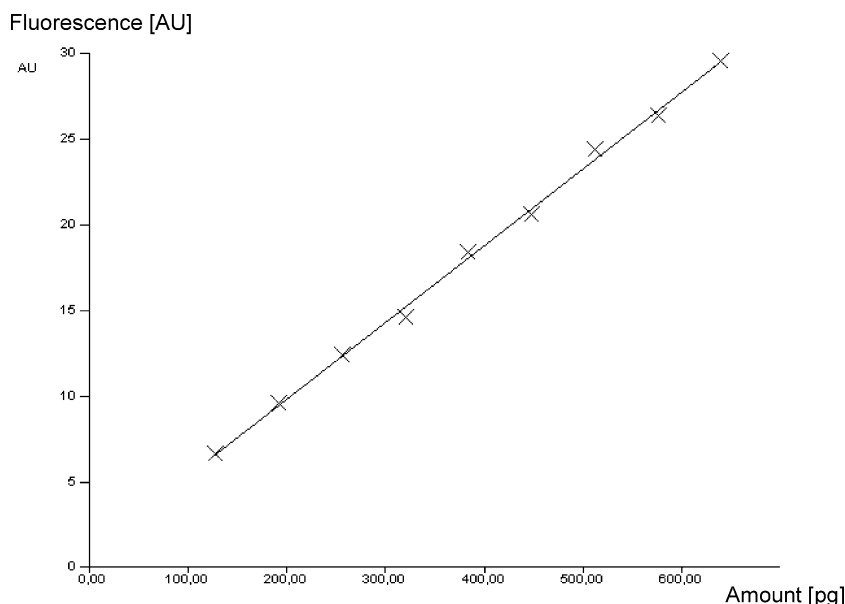


Fig. 5 Linear regression ($y = 0.045x + 0.844$) of ITX from 128 pg to 640 pg on the plate with a relative standard deviation of the calibration function of $\pm 2.18\%$ and coefficient of correlation of 0.99893 (for chromatographic conditions see Fig. 3a)



a relative standard deviation of $\pm 2.23\%$ and a coefficient of correlation of 0.99893 were obtained for ITX (Fig. 5): for DTX ($y = 0.028x + 0.694$) $\pm 1.58\%$ and 0.99945 (not shown). On reversed-phase plates, corresponding data in the range of 128 pg to 384 pg were $\pm 0.48\%$ and 0.99993 for ITX and $\pm 1.73\%$ and 0.99906 for DTX (not shown).

Limits of detection (S/N of 3) were established to be 64 pg for both ITX and DTX on the silica gel HPTLC plate. On RP18 HPTLC plates, respective limits of detection were obtained, however, on a more even baseline, i.e. less high-frequency noise, as can be seen in Fig. 6.

In food matrix, LODs were reported to be in the lower $\mu\text{g kg}^{-1}$ range via HPLC-MS [2], $10 \mu\text{g kg}^{-1}$ using GC-MS [Eurofins (2006, private communication)] and HPLC-FLD [Rothenbacher T (2005, private communication)] and $50 \mu\text{g kg}^{-1}$ via HPLC-DAD [Rothenbacher T (2005, private communication)]. Regarding HPTLC-FLD, LOD is

calculated to be $0.13 \mu\text{g kg}^{-1}$ for 100% recovery and 0.2– $2.5 \mu\text{g kg}^{-1}$ if recoveries of 6% to 70% are considered. LOD in fatty matrix, i.e. spiked butter fat, was determined to be $1 \mu\text{g kg}^{-1}$ for ITX (not shown).

Regarding robustness of the method, fluorescence stabilization (or even enhancement) and storage influence were investigated to see if a plate must be evaluated directly after chromatography. The following dipping solutions were tested for enhancement on silica gel plates: e.g. mixtures of paraffin and *n*-hexane (1:3, v/v), paraffin and toluene (1:2, v/v), Triton X100 and methanol (1:4, v/v), triethylamine and dichloromethane (1:9, v/v), PEG 2000 and methanol (1:4, v/v); however, these did not lead to any remarkable fluorescence enhancement or stabilization at all. Actually the fluorescence signal was in most cases quenched after dipping and even worse stability was obtained. Regarding the influence of storage, a plate stored in the daylight for

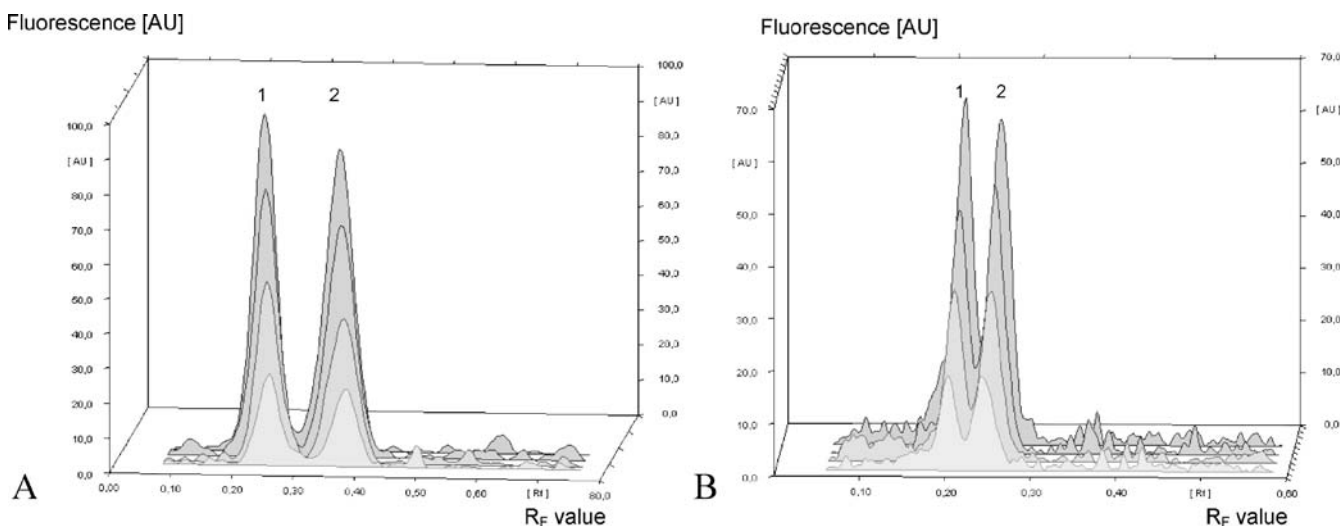


Fig. 6 Overlay of a track of 64 pg, 128 pg, 192 pg and 256 pg DTX (*peak 1*) and ITX (*peak 2*): **a** RP18 HPTLC plate, **b** silica gel 60 HPTLC plate; limits of detection (S/N of 3) for ITX and DTX were established to be 64 pg and slightly better on RP18 phases

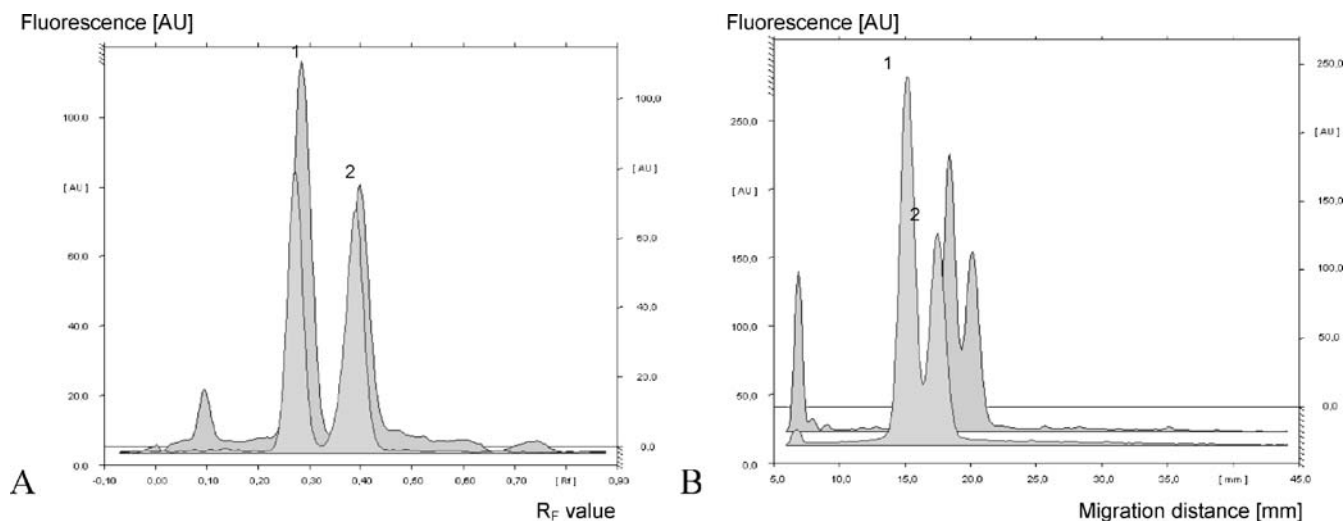


Fig. 7 Overlay of an ITX standard track (red curve) and a milk sample track (blue curve) spiked with DTX at $50 \mu\text{g kg}^{-1}$ (peak 1, internal standard) and ITX at $20 \mu\text{g kg}^{-1}$ (peak 2): **a** RP18 HPTLC plate, **b** silica gel 60 HPTLC plate (for chromatographic conditions see Fig. 3)

5 days showed a signal loss by 66 %, whereas the loss was reduced to 32 % when the plate was stored in the dark. Thus it is recommended to evaluate the plate directly after chromatography to obtain the best detection sensitivity and, if necessary, to store the plate in the dark.

A milk sample spiked with DTX (peak 1) at $50 \mu\text{g kg}^{-1}$ and ITX at $20 \mu\text{g kg}^{-1}$ (peak 2) is depicted in Fig. 7 separated on an RP18 and on a silica gel plate. It can clearly be seen that co-extracted matrix is selectively separated from DTX and ITX in both cases. On silica gel plates fluorescent matrix is fixed at the starting zone, whereas on RP phases it is slightly detectable in the lower and higher hR_F range (see also Fig. 2b, track no. 2; observable more clearly on track no. 3 albeit with a different extraction solvent); however, this did not interfere with the analysis at all. Most co-extracted matrix is not fluorescent and just causes a shift of the hR_F value which is more pronounced on silica gel phases. However as mentioned above, depending on the matrix UV-absorbing substances might quench the DTX signal in the reversed-phase system.

The migration route of ITX into the sample is not clear in detail. ITX can be present freely dissolved or associated with proteins, fat or other constituents of the sample. To imitate a real sample, migration experiments from a spiked

filter paper into milk have been simulated at maximally $200 \mu\text{g kg}^{-1}$ for 14 days at 5°C . Thereafter, ITX was clearly found with a recovery rate of about 50 %, thereby verifying the suitability of the sample preparation procedure.

Confirmation of positive findings could be performed via spectra recording in the range of 200–400 nm and comparison with a standard (identity check). Spectra identity was calculated for 3 samples each on the silica gel plates and coefficients of correlation of $r \geq 0.99974$ were obtained for ITX at 5 ng and of $r \geq 0.99984$ for DTX at 14 ng. If the ITX sample zones were wrongly assigned to the DTX standard (ITX and DTX both have similar spectra), the identity check with a default value of 0.9990 is

Table 4 Peak areas obtained by HPTLC-ESI/MS (SIR at m/z 255 and 277) for calculation of LOD and LOQ according to EURACHEM

Peak areas of SIR at m/z 255 and 277		
Mean blank value	$5,254.75 \pm 897.14$	($n=4$)
ITX (128 pg)	$16,256.00 \pm 572.57$	($n=3$)
ITX (96 pg)	$11,200.33 \pm 538.47$	($n=3$)
LOD calculated	7,946.18	
LOQ calculated	14,226.19	

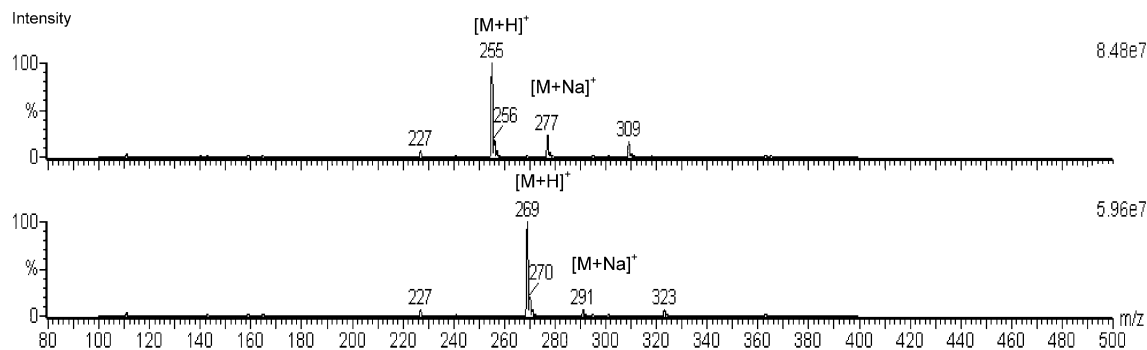


Fig. 8 Online mass spectra (ESI^+) of ITX (top) with $[\text{M}+\text{H}]^+$ at m/z 255 and $[\text{M}+\text{Na}]^+$ at m/z 277 and DTX (bottom) with $[\text{M}+\text{H}]^+$ at m/z 269 and $[\text{M}+\text{Na}]^+$ at m/z 291, both at $1 \mu\text{g}$ on silica gel plates

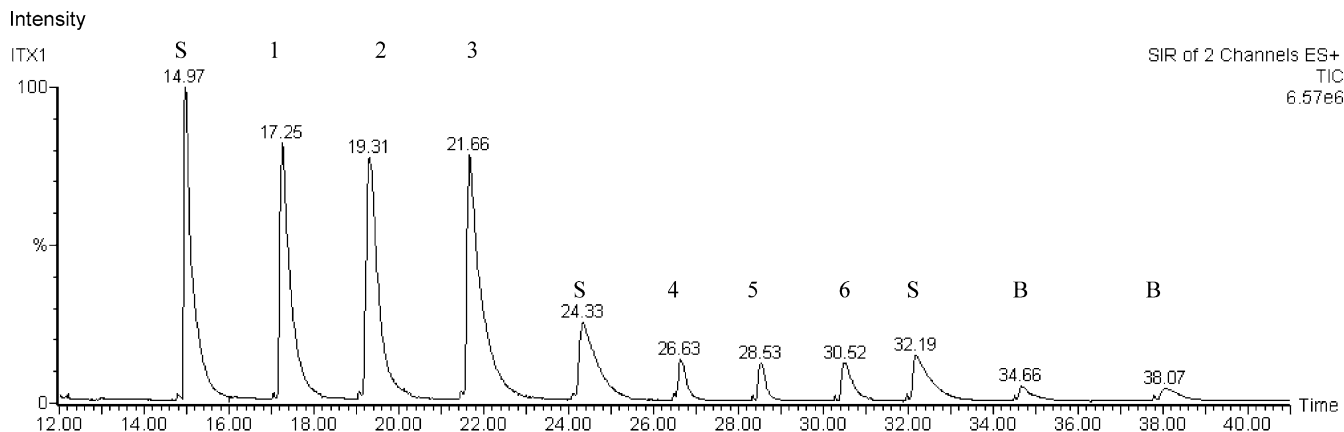


Fig. 9 Elution profile of 3 ITX zones of a yoghurt sample spiked at $100 \mu\text{g kg}^{-1}$ (peaks 1–3) and $20 \mu\text{g kg}^{-1}$ (peaks 4–6) recorded in the SIM mode at m/z 255 and 277 besides extractions of standards zones (S) and blank extractions (B)

recognizing the missing identity because coefficients of correlation for the wrong assignment were less than 0.993. This demonstrates the efficacy of the identity check in the case of substances with similar spectra.

However, more specific information on substance identity can be obtained by mass spectrometry. Mass spectra of a zone of interest could easily be recorded by means of a new online extractor [8], whose practicability and suitability was proven in trace analysis [Alpmann A and Morlock D (2006, in preparation), Jautz U and Morlock G (2006, submitted for publication)]. A modified device was used for extraction from silica gel 60 HPTLC plates. Online mass spectra in the ESI^+ mode were first recorded with high amounts ($1 \mu\text{g}$ each) to get distinct signals of ITX at m/z 255 $[\text{M}+\text{H}]^+$ and m/z 277 $[\text{M}+\text{Na}]^+$ and of DTX at m/z 269 $[\text{M}+\text{H}]^+$ and m/z 291 $[\text{M}+\text{Na}]^+$ (Fig. 8).

For determination of the minimum detectable amount, plates were pre-washed by chromatography with methanol and subsequently dried at 100°C for 30 min prior to initial use to guarantee optimal sensitivity. A signal to noise ratio of 3:1 was obtained for 128 pg ITX zones. Calculating LOD and LOQ according to EURACHEM [9] the mean value of 128 pg ITX zones ($n=3$) was slightly better than LOQ (Table 4).

For confirmation of positive findings in the $\mu\text{g kg}^{-1}$ range, ITX zones were recorded in the selective ion monitoring (SIM) mode at m/z 255 and 277 (Fig. 9). The mass spectra were recorded 4 weeks after planar chromatography was performed (plate was stored in the dark) showing robustness regarding MS measurement time of developed plates.

Further confirmation was performed by DART (direct analysis in real time) [10]. This kind of versatile new ion source works in open air under ambient conditions. The employment of DART in the field of planar chromatography was theoretically possible and its preliminary application has been demonstrated [15], however successfully, for the first time here. DART was directly coupled with a time-of-flight mass spectrometer (TOF/MS). The ITX zone on the HPTLC plate had to be exposed to a stream of excited gas and charged water clusters to allow proton transfer. The charged and desorbed sample molecules were then introduced into the vacuum system of the mass spectrometer for detection and mass spectrometric results were obtained within seconds. A prerequisite is the proper positioning of the cut plate with the analyte on an edge location. In terms of reproducibility of the signal and analytical response, a plate-positioning device would be

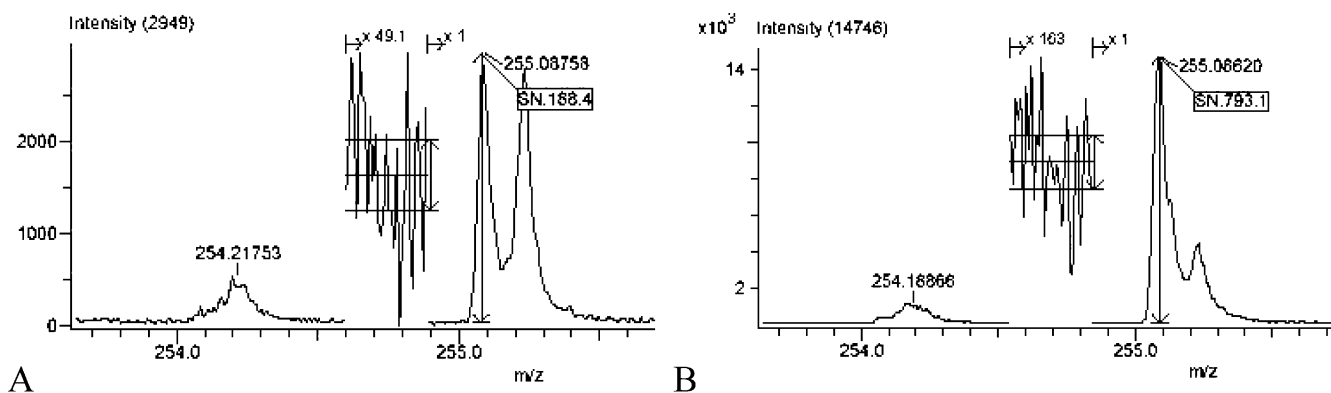


Fig. 10 HPTLC-DART/TOF spectra of a 3.2-ng ITX zone at m/z 255.08758 (a) and a 48-ng ITX zone at m/z 255.08620 (b); analytical response depends on proper positioning of the plate into the charged gas stream

desirable or working with labelled standards is generally recommended. The S/N ratio of a 3.2-ng and 48-ng ITX zone is shown in Fig. 10. The HPTLC-DART/TOF spectrum of ITX was recorded 1 day after chromatography was performed. This again showed robustness regarding time of MS measurement and development of plates.

The cost-effective employment of the MS device is worth highlighting. Not every run is recorded a priori: only positive results obtained after quantitative HPTLC can be confirmed by MS. This fact contributes to the objective to develop a rapid and cost-effective method able to handle a high sample throughput.

Conclusions

Generally planar chromatography is widely used for screening. However, the results of validation show that modern planar chromatography is highly suitable for quantification as well and can be recommended as an alternative method to quantify ITX in various matrices. Moreover the well-known features of the method, like rapidness and cost-effectiveness, contribute to a high sample throughput: in this case 36 separations last 7 min and need 10 mL solvent, meaning less than 0.2 min and 0.3 mL solvent per separation. The most time-consuming part of the procedure, the sample preparation, could be streamlined by successful employment of ASE. Both developed HPTLC methods show good results; however, particular strengths of the normal-phase separation are the slightly better reproducibility and matrix tolerance (fluorescent matrix is focussed on the starting zone) as well as the feasibility of coupling with MS and its cost-effectiveness. The presented analytical procedure can surely be

adopted to similar problems and rapid screening of other migration contaminants in food.

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