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New solvent systems for thin-layer chromatographic determination of nine biogenic amines in fish and squid

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Abstract

Different solvent systems were evaluated for their ability to separate biogenic amines by thin-layer chromatography (TLC). Dansyl derivatives of agmatine, putrescine, tryptamine, cadaverine, spermidine, histamine, spermine, tyramine and β-phenylethylamine were separated using the solvent system chloroform–diethyl ether–triethylamine (6:4:1), followed by chloroform–triethylamine (6:1). After separation dansyl amines were quantified by fluorescence densitometry at 330 nm. Correlation coefficients of linear regressions were higher than 0.99 for all amines, except for agmatine (0.976). Detection limits were 10 ng for tryptamine, tyramine, histamine and β-phenylethylamine, and 5 ng for the other amines. The overall repeatability of the chromatography was 1.82% when including agmatine and barely 1.02% for the other amines. The accuracy ranged from 105.97% (agmatine) to 49.92% (tryptamine). This thin-layer chromatography method was found to be an effective and precise analytical procedure to separate and determine biogenic amines. Its main advantages compared to previous procedures are that it uses less harmful solvent (diethyl ether instead of benzene) and can separate a larger group of biogenic amines.

Keywords: Fish; Food analysis; Biogenic amines

1. Introduction

Biogenic amines are organic bases of low molecular weight, which can be formed and degraded during the cellular metabolism in plants, animals and microorganisms [1]. Although biogenic amines are present in very low quantities in food, they can be formed during food storage. The major factors leading to their formation are probably availability of free amino acids and the presence of bacteria able to decarboxylate amino acids [2]. Although biogenic amines such as histamine, tyrosine and putrescine are needed for many essential functions in man and animals, consumption of food containing high amounts of these amines can have toxicological effects [1]. The limit for histamine content has been set at 10 mg per 100 g flesh in fish of the *Scombridae* and *Clupeidae* families at the point of first sale in the European Union (EU) [3].

Of biogenic amines occurring in fish, shellfish and derived products, the most important are cadaverine, putrescine, tyramine, histamine, spermidine, spermine, agmatine and tryptamine. Studies have shown that the production of these amines is related to fish spoilage [4–8]. Because amines are produced by spoilage bacteria, which only appear in significant numbers towards the end of the shelf-life of a fish, their concentration are more likely to be valuable as indices of spoilage rather than of freshness [3]. However, agmatine was detected at an early stage of post-mortem storage of squid of the species *Todarodes pacificus* [9] and *Todaropsis eblanae* [10] and considered excellent as a freshness indicator. In hake (*Merluccius merluccius*) stored in ice, agmatine and cadaverine may indicate freshness since they were detected before spoilage [7].

Among the different techniques recommended for identification and quantification of biogenic amines, thin-layer chromatography (TLC) and high-performance liquid chro-

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matography (HPLC) have been the most useful. Recently, the development in the field of stationary phases for TLC and instruments for automated sample application and densitometric scan has made it possible to obtain results similar to those from HPLC. TLC has many advantages, including simplicity of operation; ability to repeat detection and quantification at any time with changed parameters because fractions representing the entire sample are stored on the plate; in-system calibration for quantitative analysis; and cost effectiveness since many samples can be analysed on a single plate with use of a low amount of solvent [11].

Amines are highly polar substances that tend to streak during the chromatographic development, limiting the resolution. They are usually determined after conversion to their derivatives, which have better properties than the free amines. The most common reagent used in the derivatisation of amines is dansyl chloride. It gives highly fluorescent sulphonamide derivatives with both primary and secondary amines that are relatively stable, have improved chromatographic properties and are readily isolated from the hydrolysis product, dansyl sulphonate, by solvent extraction [12]. Different conditions have been used for dansylation of amines, i.e., 1 h at temperatures higher than 40 °C [13–16] or longer periods at room temperature [4,12,17–20]. However, to our knowledge, the effect of these different conditions on the complete conversion of amines in their dansyl derivatives has not been studied.

The separation and quantification of dansyl amines by TLC, using one dimensional development technique seems to be limited to the separation of five [16] to eight [15] amines, depending on whether single or multiple development is used. Moreover, the separation of dansyl amines can often be adversely affected by ammonia derivatives [12,16,19,20]. Dansyl-ammonia peaks are often considered well-known artefacts of dansylation reaction or consequence of impurities present in standard or samples. Because the presence of ammonia in fish muscle and consequently in fish muscle extracts is practically unavoidably, the efficiency of the diverse solvent systems in separate the dansyl amines from dansyl-ammonia must be investigated.

Several solvent systems have been evaluated and used in amine derivatives separation by TLC, and benzene is commonly used [15,18,20] despite its high toxicity.

Although some studies concern the separation and estimation of agmatine derivatives by HPLC [2,3,12,19,21], there are no studies on the determination of dansyl-agmatine by TLC. Thus, the development of a solvent system based in solvents less harmful than benzene and effective in the separation of many different amines, including agmatine, occurring in fish, shellfish and derived products would be desirable.

The objectives of this study were to evaluate different solvent systems for their ability to separate biogenic amines, including agmatine, by thin-layer chromatography, and to evaluate some of the dansylation parameters.

2. Materials and methods

2.1. Chemicals and apparatus

The biogenic amines, agmatine sulphate (AGM), cadaverine dihydrochloride (CAD), putrescine dihydrochloride (PUT), β-phenylethylamine hydrochloride (PHE) and tyramine hydrochloride (TYR) as well as 5-dimethylaminonaphthalene-l-sulphonyl (dansyl chloride) were obtained from Sigma–Aldrich, Steinheim (Germany). Histamine dihydrochloride (HIS), spermine tetrahydrochloride (SPM), spermidine trihydrochloride (SPD) and tryptamine hydrochloride (TRY) were obtained from Fluka, Steinheim (Germany). Thin-layer chromatography glass plates (20 cm × 20 cm and 10 cm × 20 cm precoated with 0.25 mm silica gel G60), high-performance thin-layer chromatography (HPTLC) glass plates (10 cm × 20 cm precoated with 0.10 mm silica gel G60) and all other chemicals and solvents were purchased from E. Merck, Damstadt (Germany).

An Automatic TLC sampler 4, twin trough chambers (21 cm × 21 cm × 6 cm) and a TLC scanner 3 programmed by Win CATS software from Camag Instruments, Muttenz (Switzerland) were used to perform the application of samples over the plate, amine separation and dansyl amines fluorescence detection, respectively.

2.2. Preparation of standards

Each amine stock standard and NH_4Cl, as a source of ammonia (AMM), was prepared at a concentration of 2.0 mg/ml (free base) in 5% trichloroacetic acid (TCA) and refrigerated until required. The work standards (20 μg/ml) were prepared by diluting 0.1 ml of the stock standards to 10 ml with 5% TCA. Mixtures of amines and AMM (range 0.25–50 μg of each compound per ml) were prepared by mixing and diluting the stock standards with appropriate volumes of 5% TCA.

2.3. Analysis of amines in fish and squid samples

Cod (*Gadus morhua*) and squid (*Illex argentinus*) were obtained from a fish market and were the freshest available. At the laboratory fish fillets and squid mantles were obtained and cut into pieces. Pieces of at least three fish or squid were packed in polyethylene bags and stored between ice layers. Samples were analysed immediately upon arrival at the laboratory and during storage to determine levels of biogenic amines. In each sampling the content of one bag (at least 50 g) was ground in a kitchen mixer. A 10 g ground sample was transferred to a Falcon tube and homogenised with 20 ml 5% TCA for 1 min using an Ultra-Turrax homogeniser. The homogenate was centrifuged (1200 × *g*, 4 min, 18 °C) and the extract filtered in filter paper. The precipitate was washed twice with 10 ml 5% TCA, centrifuged and filtered. The extracts were collected and diluted to 50 ml with 5% TCA in a volumetric flask.

2.4. Study of solvent systems for separation of biogenic amines by TLC

2.4.1. Dansylation of amines

Aliquots of 1 ml of work standards (20 μg) and mixture of standard amines and AMM (corresponding to 10 μg of each compound) were placed in test tubes and $NaHCO_3$ was added till saturation. Then, 1 ml of dansyl chloride (5 mg/ml in acetone) was added, using a Vortex mixer while adding the reagent. The tubes were closed and incubated at 40 °C for 1 h. After dansylation, 0.5 ml of 0.1 g/ml glycine solution was added to react with residual dansyl chloride. The content of tubes was mixed and tubes were heated at 40 °C for 20 min. The residual acetone was evaporated under a nitrogen stream. Water (3 ml) was added and dansyl amines were extracted with 3 × 2 ml diethyl ether, leaving the dansyl-glycine behind. The combined diethyl ether extracts were evaporated to dryness under a nitrogen stream and the residue was dissolved in 1 ml ethyl acetate. During this procedure, exposure to light was minimised.

2.4.2. Separation of dansyl amines by TLC

Using an automated TLC sampler, 10 μl of ethyl acetate containing the dansyl amine standards were applied 2 cm from the base edge of the TLC plate in 5 mm bands at 1 cm intervals. The TLC plates were not submitted to any kind of pre-treatment or activation.

Five solvent systems were evaluated for their effectiveness in separation of amines using the single development technique, and four were tested using the double development technique (Table 1). In both developments the twin trough chamber was loaded with 25 ml of the solvent system in each trough and previously equilibrated with the solvent system using a saturation pad. After developments the plate was dried and scanned at wavelength of 330 nm. The fluorescence emission of dansyl amine bands was measured using a K400 optical filter. When the double development technique was used, the plate was developed in the first solvent system and after unforced drying and scanning, the plate was developed in the same direction using the second solvent system.

Only the solvent systems 1E and 2D were evaluated for their abilities to separate PHE from the other biogenic amines.

2.5. Study of optimal reaction time and temperature for amines dansylation

Aliquots of 1 ml of mixtures of standard amines and AMM (corresponding to 10 μg and 50 μg of each compound) were placed in test tubes and $NaHCO_3$ was added till saturation. Then, 1 ml of dansyl chloride (5 mg/ml in acetone) was added, using a Vortex mixer while adding the reagent. The tubes were closed and incubated in one of five different conditions for dansylation: 30 min, 1 h, 2 h at 40 °C, 1 h at 55 °C and 16 h at room temperature. After dansylation tubes were treated as described in Section 2.4.1. The dansyl amines were applied on the TLC plates as described in Section 2.4.2 and plates were developed in chloroform–diethyl ether–triethylamine (6:4:1) for 10 cm from the start position and subsequently in chloroform–triethylamine (6:1) for 17 cm.

2.6. Linearity, precision and accuracy assays

After standardisation of dansylation and thin-layer chromatography conditions, assays were performed to determine linearity, precision and accuracy of the analytical procedure.

To determine the linearity of the proposed method standard curves for each amine were prepared within the range 0.25–50 μg/ml (eight calibration points). Standards in different concentrations were dansylated, applied on TLC plates (10 μl), separated by TLC and each dansyl amine band was densitometrically determined at 330 nm. Linear regressions, correlation coefficients, linearity range and detection limits were determined.

To determine the precision, i.e., repeatability of the analytical procedure, mixtures of amines and ammonia at

Table 1
Solvent systems evaluated for their ability to separate biogenic amines

Single development–solvent system	Ratio	Double development–solvent system	Ratio
1A–Chloroform–triethylamine[a]	4:1	2A–1st chloroform–triethylamine	4:1
		2nd diethyl ether–chloroform–acetone-triethylamine	4:6:1:1
1B–Cyclohexane–ethylacetate[b]	1:1	2B–1st cyclohexane–ethylacetate	1:1
		2nd diethyl ether–acetone–triethylamine	10:2:1
1C–Chloroform–diethyl ether–triethylamine	6:4:1	2C–1st chloroform–diethyl ether–triethylamine	6:4:1
		2nd diethyl ether: acetone: triethylamine	10:2:1
1D–Diethyl ether–acetone–triethylamine	10:2:1	2D–1st chloroform–diethyl ether–triethylamine	6:4:1
		2nd chloroform–triethylamine	6:1
1E–Chloroform–diethyl ether–triethylamine	4:1:1		

[a] Shakila et al. [16].
[b] Bencsik et al. [21].

concentrations of 5 μg/ml, 10 μg/ml and 20 μg/ml were analysed in triplicate. Averages, standard deviations (S.D.) and relative standard deviations (R.S.D.) were calculated.

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the value that is accepted as true value or an accepted reference value and the value found. It is measured as the percent of analyte recovered by assay, by spiking samples in a blind study. To determine accuracy the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline on Methodology [22] recommends collecting data from at least nine determinations over at least three concentration levels covering the specified range (for example, three concentrations, three replicates each). Thus, to determine the method accuracy, a test of recovery of amines from samples was performed. Three different amounts of the mixtures of standard amines, corresponding to 0.25 mg, 0.50 mg and 1.00 mg, were added to 10 g of ground fresh fish and squid samples. Four samples were spiked for each standard concentration. Samples were extracted with 5% TCA as described in Section 2.3. Final concentrations of the added amines were 5 μg/ml, 10 μg/ml and 20 μg/ml extract. Recoveries of amines in each concentration were calculated by comparing areas of peaks obtained for spiked fish and squid extracts with those obtained for a standard solution of the same concentration.

2.7. Statistical analysis

Results were evaluated by one-way analysis of variance (ANOVA) and Tukey test was used to detect differences between means. This statistical analysis was performed using the Statistica software, version 6.0 (StatSoft, 1995, Tulsa, OK, USA) at a significance level of $P < 0.05$.

3. Results and discussion

3.1. Amines separation

The efficiency of the solvent system in separation of biogenic amines is shown in Table 2. The solvent system 1A (chloroform–triethylamine, 4:1) had been successfully used by Shakila et al. [16] to separate PUT, CAD, HIS, SPD and TYR. However, when TRY and SPM are present, interferences in the detection of PUT and TYR, respectively, were observed. The solvent system 1B (cyclohexane–ethyl acetate, 1:1) delayed the elution of HIS, SPM and SPD, which were well separated, but all amines ran slowly. AMM interfered in CAD detection and both were close to PUT. TRY and TYR were not resolved. This solvent system was used by Bencsik et al. [21] to purify dansyl-AGM through TLC. This is the only study dealing with isolation of dansyl-AGM by TLC and reports that dansyl-AGM was positioned near the start ($R_F = 0.1$) after ascending development with the aforementioned solvent system using HPTLC plates. In the present study the dansyl-AGM remained at the start position even when the 1B solvent system was utilised jointly with HPTLC plates and in all other solvent systems evaluated as well. Using the solvent system 1C, AMM and PUT, as well as HIS and SPM were not separated, moreover, TRY, SPD and CAD remained close to each other. In the system 1D, only SPD, SPM and TYR were satisfactorily separated from the other dansyl amines. Of the solvent systems evaluated using the single development technique, the system 1E was the most effective for amine separation since only PUT and TRY were not well separated ($R_F = 0.22$ and 0.24, respectively).

Table 2
R_F values of the amines and ammonia after single development using different solvent systems[a]

Biogenic amine/AMM	Retardation factor (R_F)				
	1A[b]	1B[b]	1C[c]	1D[b]	1E[b]
Agmatine	0.00	0.00	0.00	0.00	0.00
Ammonia	0.20	0.33	0.17	0.46	0.18
Cadaverine	0.47	0.32	0.25	0.36	0.31
Histamine	0.63	0.14	0.30	0.36	0.43
Putrescine	0.34	0.29	0.17	0.32	0.22
Spermidine	0.59	0.23	0.23	0.22	0.36
Spermine	0.73	0.18	0.30	0.16	0.50
Tryptamine	0.32	0.38	0.22	0.34	0.24
Tyramine	0.71	0.38	0.48	0.53	0.58
β-Phenylethylamine	ND	ND	ND	ND	0.66

ND: not determined.
[a] Codes at the head of each column refer to solvent systems used, as described in Table 1.
[b] The solvent system was allowed to ascend for 17 cm from the start position.
[c] The solvent system was allowed to ascend for 15 cm from the start position.

Therefore, double development technique was used to achieve the amine separation by TLC, and the chromatograms are shown in Fig. 1. The system 2B represents the joint effect of systems 1B and 1D, which gave different effects in dansyl amine separation. SPM, SPD, HIS and TYR could be resolved without AMM interference, but CAD eluted together with TRY and close to PUT.

The system 2C was evaluated using a less harmful solvent as a replacement to benzene, one of the solvents utilised by Shalaby [15] to separate dansyl amines. Diethyl ether was chosen since its polarity (2.8) is similar to benzene polarity (2.7). Despite the high flammability of the diethyl ether, which requires caution, it can be regarded as a less harmful solvent in comparison with benzene. The development of the plate using the system 2C was carried out allowing the ascending of the solvents for 15 cm from the start position. The separation was inadequate and only TYR was separated from AMM and other amines.

Solvent systems 2A and 2D were based on the properties of the system chloroform–triethylamine (1A) which led to a good spread of the amines along the plate, and system

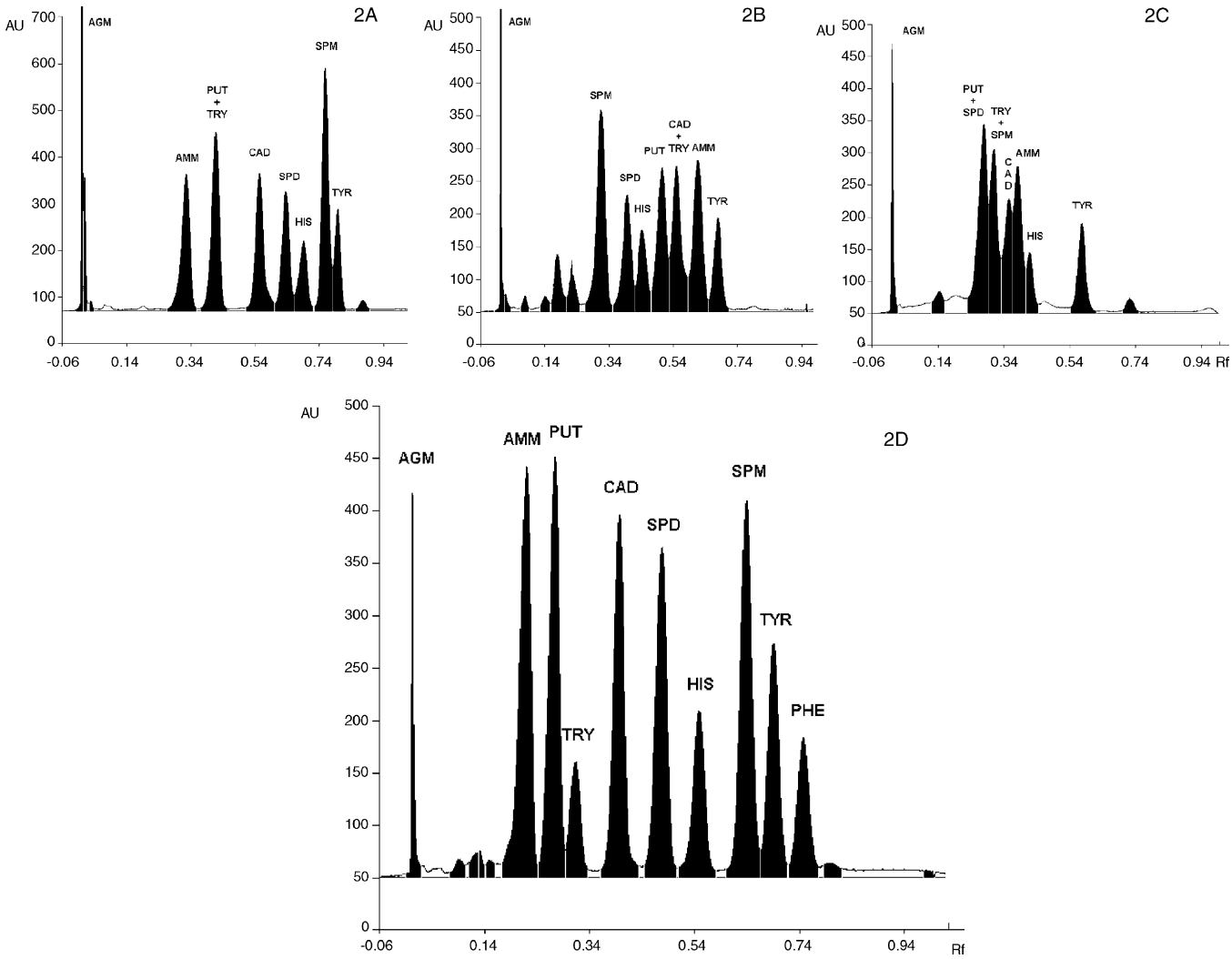

Fig. 1. Chromatogram of dansyl amines and dansyl-AMM after separation by thin-layer chromatography using double development technique. 2A, 2B, 2C and 2D are solvent systems as described in Table 1. Standards 20 μg/ml–10 μl (20 ng) applied. AGM: Agmatine; PUT: putrescine; TRY: tryptamine; CAD: cadaverine; SPD: spermidine; HIS: histamine; SPM: spermine; TYR: tyramine; PHE: β-phenylethylamine; AMM: ammonia.

1C which allowed the separation of TRY and PUT, and also SPM and TYR. In fact, the system 2A was effective in separation of SPM and TYR, but TRY and PUT remained unresolved. The system 2D was evaluated as an inversion of the sequence of solvent systems used in 2A since only system 1C was able to separate TRY and PUT and to place the others amines and AMM in a sequence that could later be well resolved by a solvent system such as chloroform–triethylamine. To obtain the best separation of amines the solvent system used during the first development was allowed to ascend on the plate for 10 cm from the start position and the solvent system used in the second development was chloroform–triethylamine, ratio 6:1. PUT, TRY, CAD, SPD, HIS, SPM, TYR and PHE were well separated, with good peak resolution and symmetry, without interference of AMM. AGM remained at the start position.

The permanence of AGM at the start position represents a serious problem for the determination of this amine by TLC since the product of the dansyl chloride hydrolysis, dansyl sulphonate, also remains at the same position. The use of glycine to react with the remaining dansyl chloride and the posterior extraction with diethyl ether are essential to eliminate the excess of dansyl chloride and dansyl sulphonate, allowing the dansyl-AGM determination. However, in food or fish samples the existence of other substances can adversely affect dansyl-AGM determination by TLC.

3.2. Dansylation time and temperature

The effects of the temperature and duration parameters on the dansylation of the amines at concentrations of 10 μg/ml and 50 μg/ml are shown in Fig. 2. For AGM, SPD, SPM and TYR when dansylation was performed for low amounts of amine (10 μg/ml) peak areas were not affected by the different dansylation conditions. However, for PUT when dansylation was performed at room temperature for 16 h,

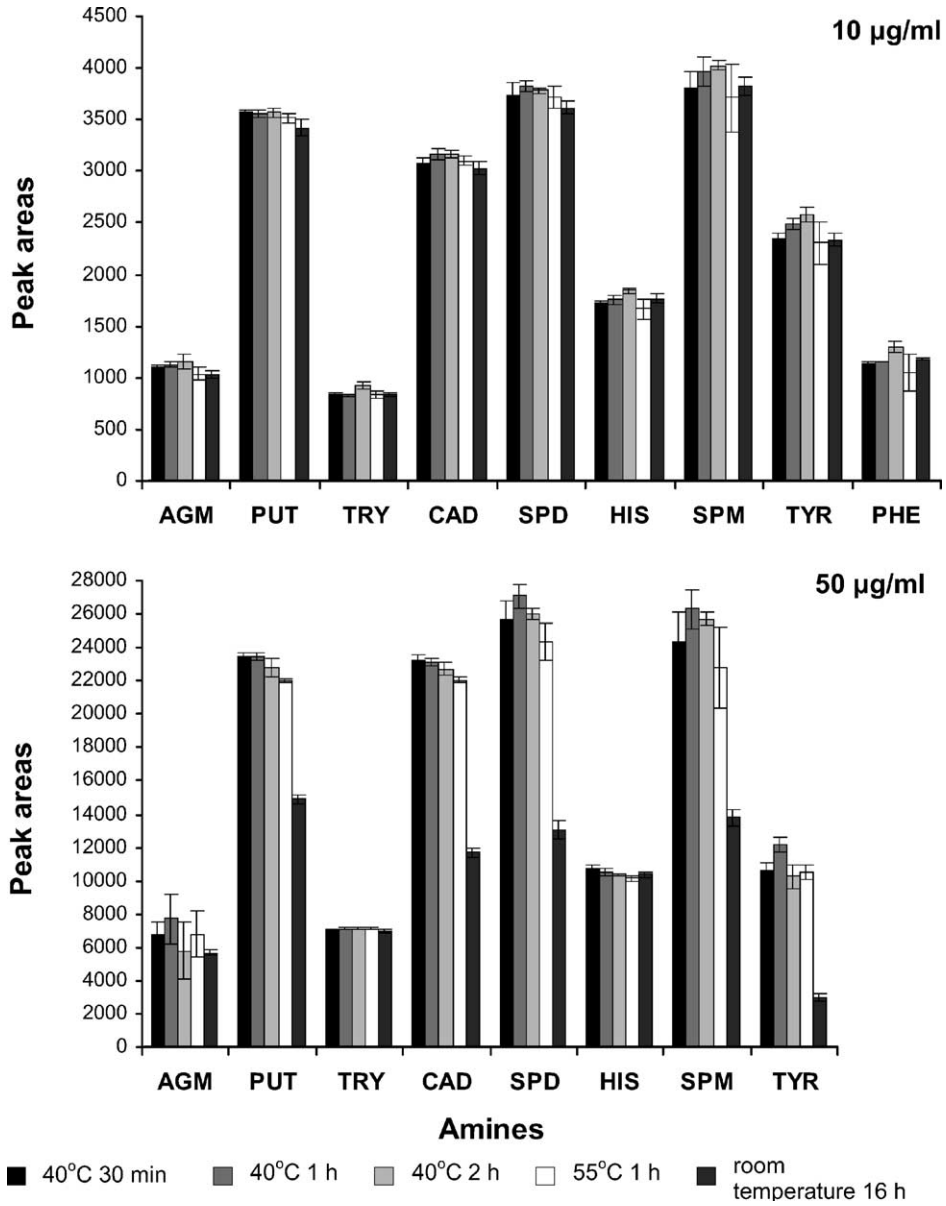

Fig. 2. Effect of temperature and duration of dansylation on the peak areas of dansyl amines. The results are presented as average value ± standard deviation. β-phenylethylamine was not evaluated at 50 μg/ml.

the peak area was significantly smaller ($P < 0.05$) than for any other condition. For PHE and HIS dansylation at 40 °C for 2 h led to greater areas than at 55 °C for 1 h. For CAD dansylation at 40 °C for 1 or 2 h led to greater areas than at room temperature for 16 h, and for TRY dansylation at 40 °C for 2 h was the best condition.

For higher amounts of amines (50 μg/ml), dansylation temperature and duration effected the formation of all dansyl amines, except AGM and TRY. Peak areas were significantly smaller ($P < 0.05$) for PUT, CAD, SPD, SPM and TYR for dansylation at room temperature for 16 h than any other condition. Dansylation at 55 °C for 1 h led to smaller peak areas than 40 °C for 30 min and 1 h for PUT and CAD, and 40 °C for 1 h for SPD and TYR.

Because dansylation at higher temperatures (55 °C) or at room temperature during a longer period (16 h) was not advantageous, statistical analysis was performed for only the effect of the dansylation duration at 40 °C. Peak areas of the amine standards allowed to react with dansyl chloride for 30 min, 1 or 2 h differed only slightly. Significant differences between 30 min and 1 h of dansylation were found only for TYR (10 μg and 50 μg) and increases ($P < 0.05$) in peak areas for 10 μg of TRY, HIS and PHE were observed doubling the reaction time from 1 h to 2 h. Thus the statistical analysis showed that AGM, PUT, CAD, SPD, SPM were completely converted in their dansyl derivatives after 30 min; TYR after 1 h; and TRY, HIS and PHE after 2 h at 40 °C. Overall RSD for amines peak areas were

Table 3
Linearity of the detector response at 330 nm and detection limits of the dansyl amines

Amine	Slope (S.D.)[a]	Intercept (S.D.)[a]	Correlation coefficient	Linearity range (μg/ml)	Detection limit (ng)
Agmatine	0.60 (0.08)	6.75 (1.36)	0.9762	0.5–50	5
Putrescine	5.82 (0.35)	2.56 (1.50)	0.9988	0.5–50	5
Tryptamine	1.69 (0.06)	5.40 (2.59)	0.9940	1.0–50	10
Cadaverine	5.60 (0.50)	8.03 (4.24)	0.9995	0.5–50	5
Spermidine	5.38 (0.30)	5.29 (2.91)	0.9992	0.5–50	5
Histamine	3.37 (0.30)	5.60 (0.50)	0.9980	1.0–50	10
Spermine	4.82 (0.04)	8.82 (2.45)	0.9997	0.5–50	5
Tyramine	3.15 (0.49)	3.72 (2.96)	0.9987	1.0–50	10
β-Phenylethylamine	2.11 (0.33)	3.46 (2.77)	0.9985	1.0–50	10

[a] Slope, intercept and standard deviation (S.D.) were multiplied by 10^{-2}.

smaller for dansylation at 40 °C for 1 h (1.78% and 4.35% for 10 μg and 50 μg, respectively) than at 40 °C for 2 h (2.31% and 5.56% for 10 μg and 50 μg, respectively). These results indicate that the optimal condition for amines dansylation is at 40 °C for 1 h since better repeatability was obtained.

3.3. Linearity, precision and accuracy assays

The linearity of the method was determined after linear regression analysis of data obtained with standard curves of each amine. The correlation coefficients were higher than 0.99 for all amines, except for AGM (0.976), show-

Table 4
Repeatability of the method for determination of dansyl amines by TLC, including dansylation and chromatography and only the chromatography step

Biogenic amine	Concentration evaluated (μg/ml)	Concentration detected (μg/ml)			
		Dansylation + chromatography		Chromatography	
		Average[a]	R.S.D. (%)	Average[b]	R.S.D. (%)
Agmatine	5	5.74	19.91	7.51	11.70
	10	12.79	9.62	7.47	8.63
	20	22.90	11.00	20.86	2.94
Putrescine	5	5.47	3.73	5.02	2.39
	10	10.68	2.85	10.80	0.65
	20	20.01	1.56	19.94	0.81
Tryptamine	5	5.35	4.49	4.99	2.04
	10	10.43	1.95	9.90	0.68
	20	19.84	0.99	19.69	2.23
Cadaverine	5	5.56	4.44	5.08	1.84
	10	10.53	2.34	10.85	1.28
	20	20.19	1.49	20.46	0.22
Spermidine	5	5.67	1.98	4.71	1.35
	10	10.42	0.76	10.12	0.10
	20	20.22	2.12	20.57	0.10
Histamine	5	6.34	7.83	4.88	1.40
	10	10.86	2.36	10.44	2.24
	20	21.59	3.84	19.57	1.72
Spermine	5	5.85	4.50	4.75	2.90
	10	10.36	1.23	10.11	0.13
	20	20.13	1.60	20.98	1.09
Tyramine	5	6.27	1.89	4.93	1.58
	10	10.73	3.54	10.42	1.23
	20	19.81	5.52	20.10	0.37
β-Phenylethylamine	5	6.29	7.76	4.50	3.63
	10	10.83	4.75	9.83	1.84
	20	21.77	4.16	19.14	1.89

R.S.D.: relative standard deviation.
[a] $n = 4$.
[b] $n = 3$.

ing that amine concentrations were linearly related with scanner responses (Table 3). The sensitivity of the method (fluorescent units per μg dansylated amine) can be determined by the relative fluorescence intensities of the dansyl amines obtained from the slopes of linear regressions. The detection limits varied with the relative fluorescence intensities and were 10 ng (1 μg/ml–10 μl applied) for TRY, TYR, HIS and PHE, and 5 ng (0.5 μg/ml –10 μl applied) for AGM, PUT, CAD, SPD and SPM. The fluorescence intensity can decrease with time due to light exposure and quenching by silica, causing a decrease in sensitivity. Thus, as it is usually recommended, standards should be applied along with samples in all TLC plates, making easy to correct any change in sensitivity by comparing the samples and standards under the same chromatographic conditions.

The effect of dansylation and chromatographic separation on overall repeatability of the method was evaluated using three different concentrations of amines (Table 4). Generally higher R.S.D. were observed for the lower standard concentration (5 μg) than higher concentrations (10 μg and 20 μg). When four samples of standards were dansylated and analysed separately, the higher R.S.D. was found for 5 μg of AGM/ml (19.91%) and the lower R.S.D. for 10 μg of SPD/ml (0.76%). The average value of repeatability for 10 μg was 3.27% including AGM and 2.47% without AGM. As expected, lower R.S.D. were generally observed when the same dansylated standard was used for the chromatog-

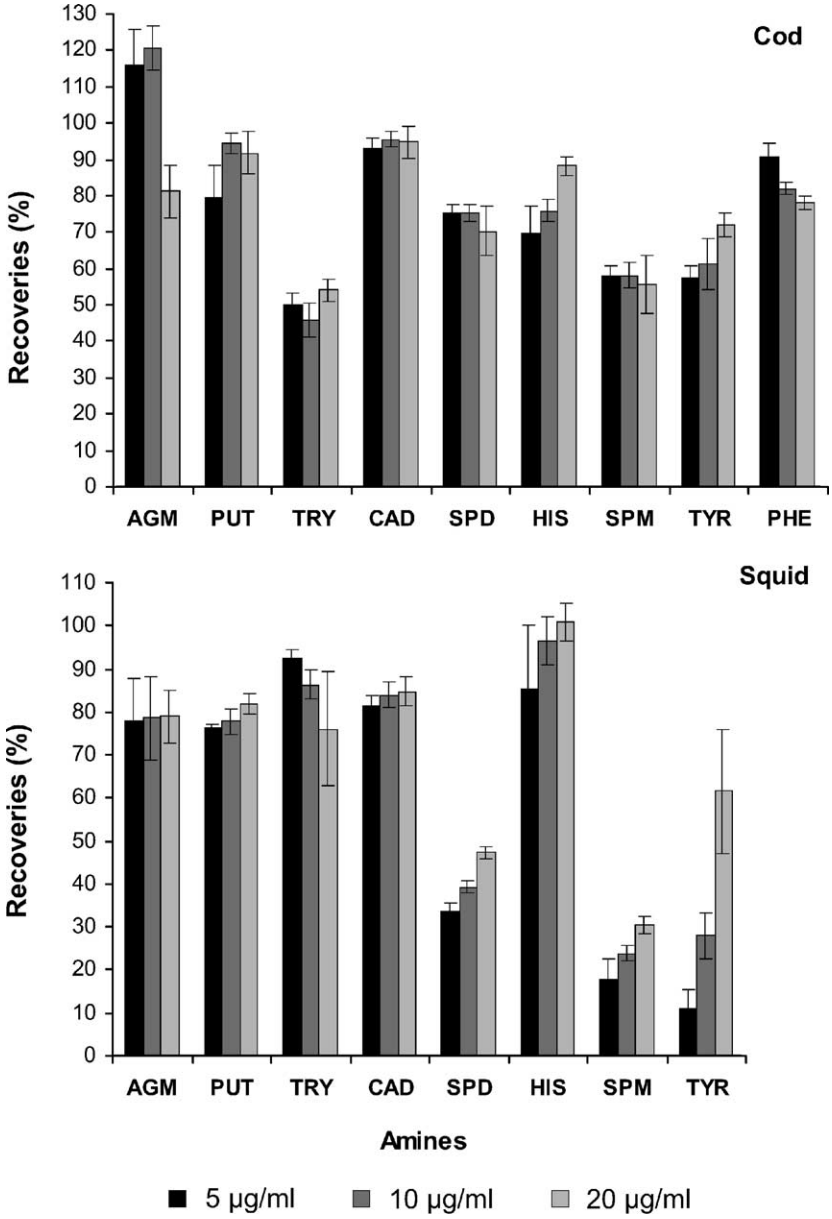

Fig. 3. Recoveries of amine standards added to cod (*Gadus morhua*) and squid (*Illex argentinus*). Three different concentrations were spiked and the final concentrations in extracts were 5 μg/ml, 10 μg/ml and 20 μg/ml. The results are presented as average value ± standard deviation.

Table 5
Levels of biogenic amines in cod (*Gadus morhua*) and squid (*Illex argentinus*) during storage in ice

Sample	Biogenic amine (mg/100 g)[a]					
	AGM	PUT	CAD	HIS	SPM	TYR
Cod						
Day 1	nd	0.09 (0.06)	nd	nd	nd	nd
Day 15	nd	1.36 (0.27)	4.87 (0.35)	nd	nd	nd
Day 25	nd	2.33 (0.05)	11.38 (0.34)	7.19 (0.94)	0.25 (0.20)	nd
Squid						
Day 1	21.58 (1.19)	nd	6.73 (0.74)	nd	nd	nd
Day 5	28.15 (2.71)	nd	14.42 (0.79)	nd	nd	19.27 (1.15)
Day 15	42.77 (2.26)	59.9 (0.67)	32.06 (1.14)	nd	nd	65.86 (2.47)

AGM: Agmatine; PUT: putrescine; CAD: cadaverine; HIS: histamine; SPM: spermine; TYR: tyramine. Tryptamine, spermidine and β-phenylethylamine were not detected. nd: not detectable.
[a] Average (standard deviation).

raphy step three times. In this case, the higher R.S.D. was again found for 5 μg of AGM/ml (11.70%) and the lower R.S.D. for 10 μg and 20 μg of SPD/ml (0.10%). The overall repeatability of the chromatography step, influenced only by sample application, TLC separation and automated peak detection was 1.82% when including AGM and barely 1.02% for the other amines, demonstrating the high precision of this analytical procedure step.

The percentages of recovery of standard amines added to fresh fish and squid samples differed depending on amine and species (Fig. 3). Also, for cod the recoveries significantly differed ($P < 0.05$) for AGM, PUT, TRY, HIS, TYR and PHE, depending on the concentration of added standard (5, 10 μg/ml and 20 μg/ml), whereas for squid recoveries significantly differed for PUT, SPD, SPM and TYR. It was not possible to find out the occurrence of similar pattern in the literature since studies on recovery of amines [14,15,18,23] evaluate the recovery of only one amine concentration. Considering the average recovery of the three concentrations, for cod the highest recoveries were found for AGM (106.0%), CAD (94.4%), PUT (88.6%) and PHE (83.7%), intermediate recoveries for HIS (77.9%), SPD (73.5%) and TYR (63.6%) and lowest recoveries for TRY (49.9%) and SPM (57.4%). For squid the highest recoveries were found for HIS (94.3%), TRY (85.1%) and CAD (83.4%), intermediate recoveries for PUT and AGM (78.6% for both) and the lowest recoveries for SPD (40.2%), TYR (33.5%) and SPM (24.1%). Moret and Conte [14] and Hwang et al. [23] also observed considerable variation among the percentages of recovery of amines added to fish samples.

Studies have shown that TCA solution is the best solvent for extraction of amines from meat and meat products [24] and from fish muscle [14]. However, affinity between amine and protein matrix, and small differences in the extraction procedure (sample: TCA solution ratio, degree of homogenizing and protein precipitate separation) seem to drastically influence the amines partition and consequently the amine recoveries.

For AGM it is important to point out that the high percentage of recovery in cod, over 110% for 5 μg/ml and 10 μg/ml, may indicate that other substances present in the fish extract, which also remained at start position after TLC separation, can adversely affect AGM determination.

3.4. Determination of amines in fish and squid samples

Levels of biogenic amines in cod and squid were determined during storage in ice to evaluate the applicability of the TLC method with the solvent system chloroform–diethyl ether–triethylamine (6:4:1) followed by chloroform–triethylamine (6:1) (Table 5). PUT was the only biogenic amine present in fresh cod and its levels progressively increased during storage. After 15 days of storage CAD was detected in higher levels than PUT. After 25 days of storage, when unequivocal signs of spoilage were observed in cod, SPM was detected in low concentration and CAD and HIS were present in high concentrations. Hernández-Herrero et al. [25] reported that the biogenic amine contents in gutted cod varied considerably during iced storage, and concluded that freshness indices based in these substances are not useful to evaluate quality of cod. The results obtained in the present study indicate that the contents of some biogenic amines in cod probably correlate with storage time, which deserves a further investigation. In squid AGM and CAD were detected in high concentrations as early as the first day and their concentrations increased during storage. TYR was observed from day 5. After 15 days of iced storage, when unequivocal signs of spoilage were observed in squid, PUT and TYR were determined in concentrations higher than 50 mg/100 g. This increase in AGM and CAD levels during the storage of squid (*I. argentinus*) indicates that these amines could be good quality indices for this squid species. AGM, CAD, PUT and TYR were also the main biogenic amines detected by Yamanaka et al. [9] after squid (*T. pacificus*) was stored 14 days at 0 °C, and the authors suggested AGM as good freshness index.

4. Conclusion

One dimensional, double development technique, using the solvent system chloroform–diethyl ether–triethylamine (6:4:1) followed by chloroform–triethylamine (6:1) was found to be an effective and precise analytical procedure for separation and determination of the dansyl derivatives of AGM, PUT, TRY, CAD, SPD, HIS, SPM, TYR and PHE by TLC. Besides the advantages concerning costs, simplicity and versatility, the main advantages of this TLC methodology compared to previous procedures are that it uses a less harmful solvent (diethyl ether instead of benzene) and can separate nine biogenic amines. The use of this method for dansyl-AGM determination should be considered with caution, since substances present in sample extracts can cause interference, resulting in overestimated AGM values.

One dimensional, single development technique using the solvent system 1E (chloroform–diethyl ether–triethylamine, 4:1:1) can be a good and even simpler option for separation and determination of eight dansyl amimes by TLC. The linearity, precision and accuracy of the TLC methodology using this solvent system, as well as the applicability of these procedures for determining amines in food in general, should be further investigated.

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