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Analytical Methods

Dabsyl derivatisation as an alternative for dansylation in the detection of biogenic amines in fermented meat products by reversed phase high performance liquid chromatography

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ABSTRACT

The commonly applied HPLC method to determine biogenic amines in dry fermented meat after dansylation was compared with an alternative dabsylation procedure. The use of dabsyl chloride at 70 °C resulted in a 25-min reduction of the derivatisation time, in comparison with the dansylation at 40 °C. Furthermore, the use of irritating ammonia to remove the excess of dansyl chloride can be avoided. Introduction of the SPE cleaning procedure on the C18 cartridge resulted in a reliable and sensitive method of biogenic amines determination in a complex protein–fat matrix, which is typical of dry fermented sausages.

The biogenic amines tryptamine (TRYP), phenethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonin (SER), tyramine (TYR), and the natural polyamines spermidine (SPD) and spermine (SPM), were separated by means of gradient HPLC, using the two coupled C18 Chromolith reversed-phase columns.

1. Introduction

Biogenic amines (BAs) are basic nitrogenous compounds, formed mainly by microbial decarboxylation of the free amino acids in a foodstuff (Karovičová & Kohajdová, 2005). Fermented foods can certainly be considered as a reservoir for accumulation of BAs. This is because the fermentation process gives the predominant microbial flora an opportunity to decarboxylate the increasing amount of free amino acids (Lorenzo, Martínez, Franco, & Carballo, 2007).

In the dry fermented sausages, the main biogenic amines are tyramine (TYR), putrescine (PUT), cadaverine (CAD), spermine (SPM) and spermidine (SPD), and to a lesser extent, phenethylamine (PHE), histamine (HIS), tryptamine (TRYP), and serotonin (SER) (Ruiz-Capillas & Jiménez-Colmonero, 2004). Since high amounts of these organic substances can induce toxicological risks and health problems, monitoring of their levels is very important. Hence, several analytical procedures for the determination of BAs in food samples have already been elaborated.

One considerable difficulty is the isolation of BAs from a complex matrix of the food sample. Solid samples are most frequently extracted with acidic solvents which also act as deproteinisation agent during the liquid solid extraction (LSE). While hydrochloric acid is used for the extraction of cheese samples, it is not recommended for the extraction of meat and fish, because of the possible occurrence of turbidity (Innocente, Biasutti, Padovese, & Moret, 2007). In the meat and fish analysis, trichloroacetic acid (TCA) (Ferreira et al., 2006; Masson, Johansson, & Montel, 1999) or, more frequently, perchloric acid ($HClO_4$) (Dadáková, Křížek, & Pelikánová, 2009; Eerola, Hinkanen, Lindfors, & Hirvi, 1993; Latorre-Moratalla et al., 2008) is used. Most research dealing with solid food samples limits the sample clean-up to LSE, although the resulting extracts still contain several interfering compounds. To remove these compounds and concentrate the sample, solid phase extraction (SPE) can either be used after the LSE procedure, or directly as the matrix solid phase dispersion (MSPD) (Calbiani et al., 2005). Strong cationic-exchange cartridges (SCX) or silica adsorbents are mainly used for the extraction of aliphatic and biogenic amines. However, the difference in the pK_a-values of amines makes it difficult to simultaneously elute the target analytes from the adsorbent

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and the use of ion-pair reagents is often required (Molins-Legua & Campins-Falcó, 2005). Another possibility to enhance the coelution of the target compounds is the SPE sample purification on a C18 cartridge after derivatisation (Soufleros, Bouloumpasi, Zoutou, & Loukou, 2007).

Amongst the available analytical techniques, HPLC is by far the most frequently used to separate and quantify BAs. Since most BAs present in the food samples neither show an adequate absorption, nor exhibit significant fluorescence, derivatisation has to be performed in order to increase the sensitivity needed for a subsequent UV, VIS or fluorescence detection (Önal, 2007). The use of an HPLC system equipped with an UV detector requires derivatisation with a chromophoric reagent such as dansyl-chloride (Dns-Cl) (Dadáková et al., 2009; Saarinen, 2002), or dabsyl chloride (Dbs-Cl) (Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995; Romero, Bagur, Sánchez-Viñas, & Gázquez, 2003). Although dansyl chloride is the most widely used derivatisation reagent in the analysis of BAs by means of RP-HPLC–UV, this method demonstrates certain drawbacks, when applied to the determination of BAs in food samples. One drawback is the long derivatisation time, and another one is that the alkaline ammonia has to be used to remove the interfering by-products. In this study, the dansylation procedure (Eerola et al., 1993) is compared with the closely related, yet faster dabsylation procedure. Although dabsylation is less frequently applied in meat analysis, its advantage over the predominantly used dansyl chloride reagent is that dabsyl derivatives show absorbance in the range of 436–460 nm. In that way, interferences from UV-absorbing biological compounds present in the meat extracts are mostly avoided (Aboul-Enein, 2003). For dabsylation of biogenic amines in several foodstuffs, small variations amongst the methods can be found (Castillo & Castells, 2001; Krause et al., 1995; Romero, Bagur, Sánchez-Viñas, & Gázquez, 2000). Therefore this study is carried out to determine the critical parameters affecting the derivatisation yields of the aforementioned BAs. An ultimate goal is to develop a reliable and robust method for the detection and quantification of several biogenic amines in the meat products.

2. Experimental

2.1. Preparation of the amine standard solutions

The standards (either in the form of the free bases, or the respective hydrochlorides), i.e. SPM, SPD, CAD, PUT, PHE, TYR, SER, HIS and TRYP were purchased from Sigma Aldrich (Bornem, Belgium). The mixed stock solution, containing 1000 $\mu g \cdot mL^{-1}$ of each individual amine, was prepared by dissolving adequate amounts of the amines in a mixture of methanol and 1 M HCl (1:1, v/v) (both chemicals purchased from VWR International, Leuven, Belgium). A separate internal standard (IS) stock solutions, containing 1000 $\mu g \cdot mL^{-1}$ 1,7-diaminoheptane (Sigma Aldrich), was made in an analogous way. These solutions were stored for periods of up to one month at the temperature −28 °C. Seven working solutions, at the concentrations of 0.08, 0.40, 0.80, 2.00, 4.00, 6.00, 8.00 $\mu g \cdot mL^{-1}$ of each individual amine, respectively, were obtained from the stock solution by an appropriate dilution with 0.4 M $HClO_4$ (VWR International). These amine mixtures were stored at 4 °C for one week. The IS stock solution was diluted with 0.4 M $HClO_4$ to the concentration of 8 $\mu g \cdot mL^{-1}$, in order to obtain an IS working solution.

2.2. Liquid–solid extraction of the meat sample (LSE)

The 2-g aliquot of a finely cut dry fermented meat sample was weighed and then spiked with 200 μL of IS working solution. The 10-mL aliquot of 0.4 M $HClO_4$ was added and the entity was homogenised with an Ultra-Turrax T18 homogeniser (IKA, Staufen, Germany). The extracts were stored at 4 °C to crystallise the fat. The meat sample was centrifuged (Heraeus Labofuge 200, Fisher Scientific, Tournai, Belgium) for 10 min at 1000 g and the upper fat layer was removed. Subsequently, the extraction was repeated for the second time. The filtered supernatants were combined and the resulting volume was made up to 25 mL with 0.4 M $HClO_4$.

2.3. Derivatisation

2.3.1. Dansylation

The 2-mL aliquot of the amine mixture, or sample extract was transferred to the 10-mL test tube, and the pH was adjusted to 9.5–10.0 by adding 400 μL 2 M NaOH and 600 μL buffer solution (0.95 M $NaHCO_3$). The dansyl chloride solution was freshly prepared each day by ultrasonic dissolution of 10 mg Dns-Cl (1-dimethylamino-naphtalene-5-sulfonyl chloride, Sigma Aldrich) per 1 mL acetonitrile (Fisher Scientific). The 4-mL portion of dansyl chloride solution was added to the test tube and then thoroughly vortexed. Following the procedure elaborated by Eerola et al. (1993), derivatisation was carried out for 45 min at 40 °C. After this incubation period, the excess of dansyl chloride was removed by adding a portion of concentrated ammonia (VWR International) and incubating the test tube at room temperature in the dark. In order to obtain a chromatogram without an interference of Dns-Cl, three different volumes (i.e. 0, 100 and 200 μL) of ammonia and two different incubation times (i.e. 30 and 60 min) were tested.

2.3.2. Dabsylation

An analogous volume of the extract as for dansylation (i.e. 2 mL) was used for the derivatisation with dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride, Sigma Aldrich). To study the optimal pH value for dabsylation, portions of 2 M NaOH varying from 150 to 500 μL were added to cover the pH range from 7.4 to 10.6. Buffering of the samples was done by adding 600 μL of 0.95 M $NaHCO_3$. The 4-mL portion of the dabsyl chloride solution (5 mg Dbs-Cl per 1 mL acetonitrile) was added to the sample. Incubation was performed at a temperature of 70 and 80 °C for 20 min, in order to establish a better dabsylation yield. Ultimately, the reaction was stopped by cooling the test tubes in an ice bath for 30 min.

2.4. Sample purification and concentration

2.4.1. Liquid–liquid extraction (LLE)

Three times, a 2-mL portion of diethyl ether (Fisher Scientific) was added to the derivatised sample and shaken for 1 min. Each time, after separation of the two phases, the upper organic layer was transferred to the second test tube. Ultimately, the three organic fractions were combined and evaporated to dryness at 30 °C under a steady stream of nitrogen.

2.4.2. Solid-phase extraction (SPE)

To obtain good recovery of the biogenic amines, the experiment was set up to determine the best possible conditions of carrying out the SPE procedure. Therefore the different eluted fractions were collected separately and each one was analysed for the target compounds. In the first experiment, SPE purification of the sample was directly applied after the perchloric acid extraction. The Gracepure C18 cartridge (1000 $mg \cdot 6\ mL^{-1}$) (Grace Davison Discovery Sciences, Lokeren, Belgium) was activated with 6 mL acetonitrile and equilibrated with 6 mL ultra pure water. The extract was loaded on top of the cartridge and washed twice with two 4.5-mL portions of ultra pure water. Elution of the biogenic amines was forced with the three 4.5-mL portions of acetonitrile. In the

second experiment, the sample was first derivatised with dabsyl chloride and then treated as in the first experiment. In the third experiment, the C18 cartridge was subsequently activated and conditioned with 6 mL acetonitrile and 6 mL 0.4 M $HClO_4$. The dabsylated extract was loaded on the SPE cartridge and washed with two 4.5-mL portions of 0.4 M $HClO_4$. Finally, the amines were eluted with the three consecutive 4.5-mL portions of acetonitrile. The obtained fractions were concentrated under nitrogen at 35 °C with the use of the TurboVap workstation (Caliper Life Sciences, Teralfene, Belgium).

2.4.3. Sample dilution

After the evaporation to dryness, the residue had to be dissolved in an appropriate solution to prepare the sample for chromatographic analysis. Originally the extract was liquefied in 5 mL mobile phase A (used at the start of the gradient sequence), which was a mixture of methanol, acetonitrile and water (12.5:37.5:50, v/v/v). Then dilution buffers, composed of acetonitrile, ethanol and elution buffer (9 mM NaH_2PO_4) (50:25:25, v/v/v) at the different pH values between 2.0 and 12.5 were made to study the influence of the stability of the extracts.

2.5. Chromatographic procedure

HPLC was carried out on a Hitachi La ChromElite (VWR International) with UV detector. Prior to use, the two coupled Chromolith®Performance RP-18e columns (100 × 3 mm i.d., VWR International) were equilibrated for 24 h with 100% mobile phase A (methanol:acetonitrile:water; 12.5:37.5:50, v/v/v) and thermostated at 40 °C during the analysis. The 10-μL aliquots of the derivatised extract were chromatographed at a flow rate of 1.0 mL·min^{-1}. For the dansylated amines, the following gradient profile was used: From 0% to 25%, mobile phase B (methanol:acetonitrile, 25:75, v/v) in 5 min, 25% for 5 min, an increase to 55% in 6 min, a jump to 100% in 1 min, 100% for 4 min, then a decrease to 0% in 2 min, and finally equilibration at 100% mobile phase A for 3 min. For the less polar dabsyl derivatives, the gradient profile was adjusted, as follows: From 0% to 40%, mobile phase B in 8 min, increase to 60% in 5 min, raise to 100% in 7 min, 100% for 5 min, decrease to 0% in 2 min, and finally equilibration at 100% mobile phase A for 3 min. The detection wavelength was 254 nm for the dansyl derivatives and 450 nm for the dabsyl derivatives.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation (n = 6 or 8). The linear least-square regression was used to calculate the intercepts (a), slopes (b) and the coefficient of determinations (R^2) of the calibration curves (Microsoft Office Excel 2007, Microsoft Corporation, Redmond, WA). To determine the matrix effect, a t-test was performed to compare the slopes of the calibration curves.

3. Results and discussion

3.1. Derivatisation

3.1.1. Dansylation

Although the dansylation procedure – as described by Eerola et al. (1993) – is widely used for determination of biogenic amines in all kinds of food samples, its evident drawback is the long derivatisation time. Moreover, ammonia has to be used after the derivatisation in order to remove an excess of the dansyl reagent. Without this step, the by-products such as dansylamide (Dns-NH_2), dansyl sulphonic acid (Dns-OH) and dansyl hydrazine (Dns-N_2H_3) most likely appear in the chromatogram (marked as * in Fig. 1). Moreover, an excess of dansyl chloride coelutes with putrescine. Unfortunately, an addition of 100 μL concentrated ammonia and incubation for 30 min at room temperature, as suggested by Eerola et al. (1993), could not totally remove the excess of dansyl chloride. As a result, low amounts of the by-products can still be detected in the chromatogram and the peak of putrescine does not remain unaffected. Doubling the amount of added ammonia or doubling the incubation time did not affect the occurrence of these by-products.

3.1.2. Dabsylation

In contrast to the dansylation procedure, dabsylation is far less frequently used for the determination of biogenic amines in dry fermented meat products. However, fast dabsylation gives an opportunity to reduce the analysis time. From the literature it

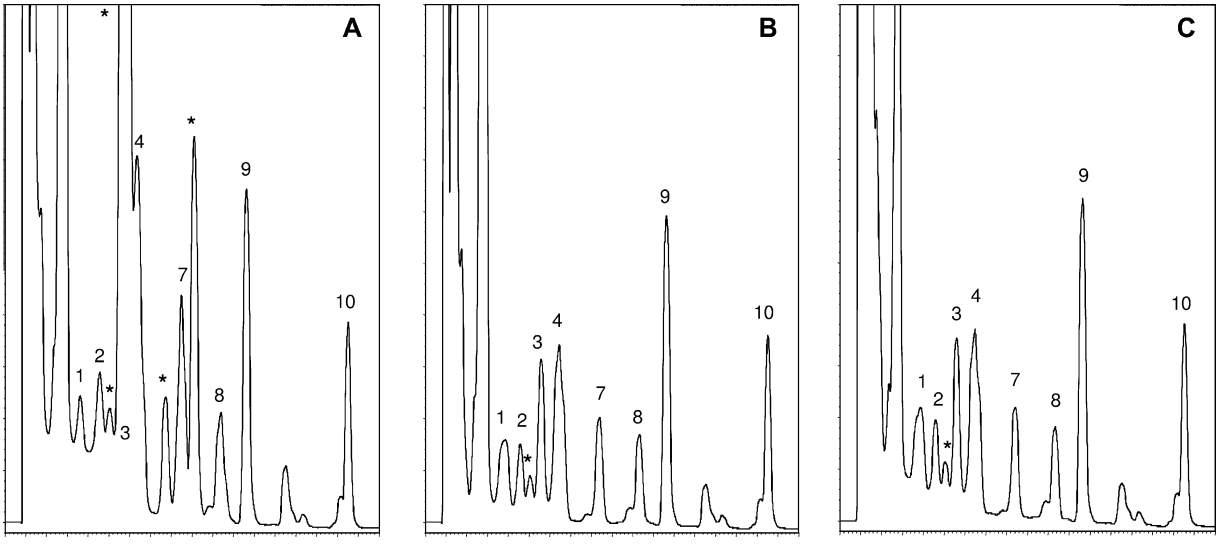

Fig. 1. Chromatograms of three aliquots of the same spiked dry fermented sausage sample, derivatised with dansyl chloride and the addition of three different volumes of ammonia; i.e. (A) 0, (B) 100, and (C) 200 μL. Identification of the peaks: (1) TYR, (2) PHE, (3) PUT, (4) CAD, (5) HIS, (6) SER, (7) IS (1,7-diaminoheptane), (8) TYR, (9) SPD, (10) SPM.

comes out that the dabsyl derivatisation used to be performed at the temperatures ranging from room temperature to 70 °C. At the room temperature, the derivatisation needs an overnight incubation, while at the increased temperatures the incubation time can range between 15 (Krause et al., 1995) and 30 min only (Chen, Shih, Liou, & Chen, 2003). In most cases, an incubation period of ca. 20 min at 70 °C is recommended (Romero et al., 2000). In contrast to dansylation (which shows severe decomposition of dansylated amines at the temperatures higher than 65 °C), Dadáková et al. (2009) and Castillo and Castells (2001) have mentioned that at higher temperatures the peak ascribed to the excess of dabsyl reagent decreased, without the loss of the peak areas of the analytes. In this study, we therefore investigated, if dabsylation could be carried out at 80 °C in order to reduce the dabsyl chloride peak. As it comes out from our investigation, the derivatisation temperature could not be set at 80 °C without the loss of the analytes. The total peak area was by 37% lower, especially due to the reduction of the peaks originating from histamine (96%) and spermine (79%). Although the peak of the excess of dabsyl reagent could not be completely eliminated from the chromatogram, we have ultimately decided to employ 70 °C as an incubation temperature and the test tubes were shaken after 1 and 15 min of incubation, as recommended in the literature (Romero et al., 2000). In that way, the highest available yields of the studied analytes were obtained and as the excess peak of the dabsyl reagent was separated from the analytes, no problems were encountered with quantification thereof.

In contrast to the dansylation (pH between 9.5 and 10.0), according Romero et al. (2000) the pH range for the dabsyl reaction has to be set between 8.0 and 8.5, with an optimal value of 8.2. Other authors mention buffering of the reaction solution at pH 8.6 (Krause et al., 1995), and even at 9.2 (Castillo & Castells, 2001). To check, if the pH range for the derivatisation is as critical, as described by Romero, we carried out an investigation in order to determine the pH range for robust derivatisation. As it can be seen from Fig. 2, derivatisation running under almost neutral pH (e.g. at pH 7.4) has to be avoided. The impact of the pH value on the total peak area and on the peak areas of the individual amines is negligible in the pH range from 8.0 to 10.0. For HIS only, an evident pH optimum of 8.6 could be detected. Therefore, we decided to set the pH at ca. 8.6 by adding 300 μL 2 M NaOH and 600 μL buffer solution to the reaction mixture.

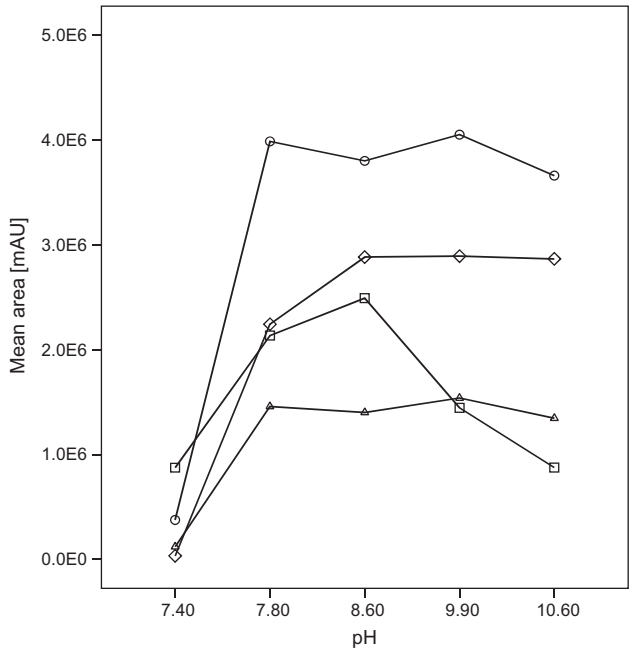

Fig. 2. Influence of the pH value of the reaction mixture on the dabsyl derivatisation yield at 70 °C with SPD (○), TYR (◇), HIS (□) and SPM (Δ).

Another advantage of using dabsyl chloride is the simple way to stop the derivatisation reaction. While an addition of irritating ammonia is necessary to remove an excess of dansyl chloride, an easy cooling step, i.e. an ice bath for 30 min, is sufficient to stop the dabsylation reaction.

3.2. Sample purification

3.2.1. Liquid–liquid extraction

The method of Eerola et al. (1993) does not include additional sample purification, but such steps ought to be considered when analysing complex food matrices, in particular dry fermented sausage samples. To determine low concentrations of biogenic amines in the protein rich matrices, it can be of great importance to remove interfering compounds from the chromatogram with the intention to obtain the baseline separated peaks. Therefore an additional liquid–liquid extraction with diethyl ether was employed, in order to remove highly polar or ionic compounds from the meat extract. Using three 2-mL portions of diethyl ether was sufficient to extract all BAs. Although the amino acids successfully remained in the aqueous phase, no great improvement of the chromatograms was observed. Diethyl ether was probably not selective enough to extract the dansylated amines from the reagent impurities which still interfered in the chromatogram.

3.2.2. Solid phase extraction

As LLE proved unable to result in good quality chromatograms and appeared time consuming and insufficiently repeatable, the SPE technique was alternatively introduced to provide an extra clean-up. Due to the characteristics of the sample, i.e. high percentage of proteins and ionic compounds, the Gracepure C18-max cartridge with a high carbon percentage (17.1%) was selected. In the course of optimisation of the SPE procedure, the different fractions (loading, washing and elution) were separately collected from the cartridge, and their composition was analysed in order to examine the distribution of the target compounds in the effluents.

In the first experiment, the SPE purification of the sample was directly applied after the perchloric acid extraction. The cartridge was activated with 6 mL acetonitrile and equilibrated with 6 mL water. It can, however, be seen from Fig. 3 (A, Experiment 1) that the biogenic amines were distributed over all the fractions, due to the great polarity differences of the underivatised BAs. To overcome this problem, the BAs were derivatised prior to the application of the SPE procedure (Soufleros et al., 2007). In that way, the analytes became more polar and were better retained in the course of washing. As shown in Fig. 3 (A, Experiment 2), after modification of the original procedure, the greater part of the biogenic amines was collected in the elution fractions. However, a small percentage was still eluted in the loading and the first washing fraction. The main reason was an inadequate retention of PHE (Fig. 3, B) and TRYP (data not shown). Finally, the retention of all the amines during the washing phase was guaranteed when water (used as the equilibration/washing solvent) was replaced with 0.4 M $HClO_4$ (Fig. 3, B, Experiment 3). Moreover, addition of 0.4 M $HClO_4$ improved the elution and only two 4.5-mL portions of acetonitrile were enough to elute all biogenic amines. Similar to the extraction with diethyl ether, acetonitrile as the elution solvent was evaporated to concentrate the extract.

3.2.3. Sample dilution

Dissolution of the dry residue was first made in 5 mL mobile phase A (methanol:acetonitrile:water, 12.5:37.5:50, v/v/v), but

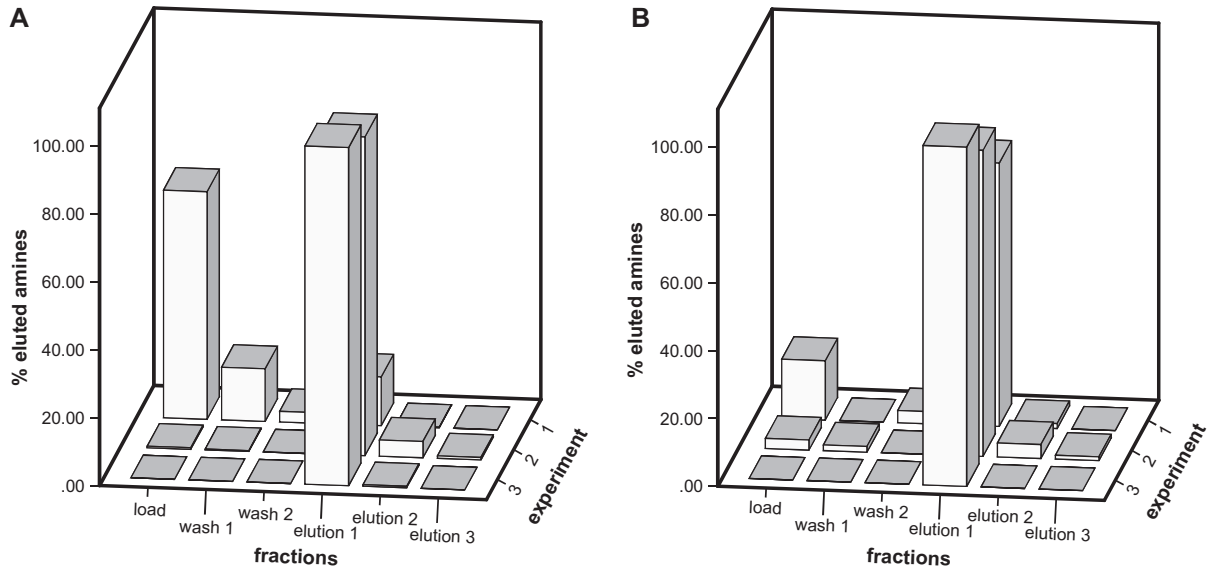

Fig. 3. Distribution of the target amines; (A) total BAs, and (B) PHE amongst the different fractions collected during the SPE purification; Experiment 1: 6 mL underivatised extract, washed with 2 × 4.5 mL water and eluted with 3 × 4.5 mL acetonitrile, Experiment 2: 6 mL dabsylated extract, washed with 2 × 4.5 mL water and eluted with 3 × 4.5 mL acetontrile, Experiment 3: 6 mL dabsylated extract, washed with 2 × 4.5 mL 0.4 M $HClO_4$ and eluted with 3 × 4.5 mL acetonitrile.

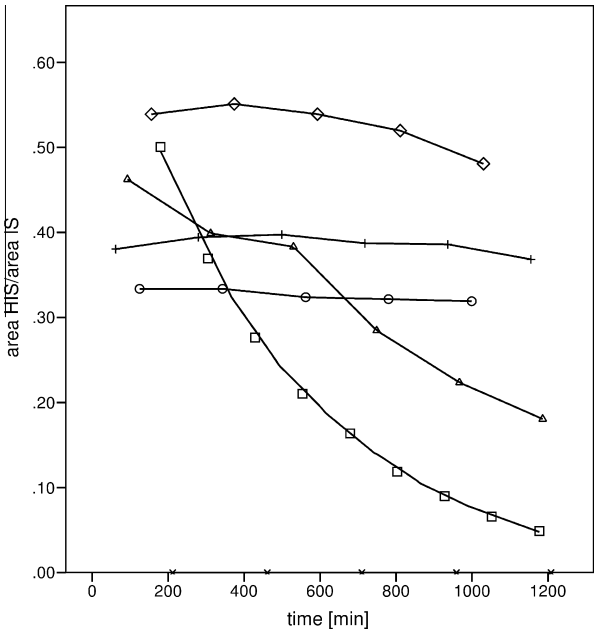

Fig. 4. Degradation of dabsyl-HIS (area HIS/area IS) in time, buffered in a diluting solution (acetonitrile:ethanol:dilution buffer (9 mM NaH_2PO_4), 50:25:25, v/v/v) and adjusted with 2 M NaOH at pH 2.0 (□), 3.7 (Δ), 4.0 (○), 7.3 (◇), 7.6 (+) and 12.5 (×).

dissolved HIS seemed unstable at room temperature, when kept for 24 h. Krause et al. (1995) suggested using of a dilution buffer to overcome the crystallisation problem of certain BAs (such as HIS and TYR) during a prolonged storage time. This recommended dilution buffer was composed of acetonitrile, ethanol and elution buffer (9 mM NaH_2PO_4) (50:25:25, v/v/v). The use of this solution (buffered within the pH range from 2.0 to 12.5) showed that the stability of histamine could be improved, when the extract was buffered at ca. pH 7 (Fig. 4). However, the introduction of the dilution buffer – regardless of the applied pH value – induced instability of SPD and the yield of SPM could even be reduced to zero. Therefore it was finally decided to keep the mobile phase A as the dry residue solvent. The stability of HIS could be ensured by keeping the HPLC vials at 4 °C until the analysis time.

3.3. HPLC separation

Initially a simple gradient program starting with 50% 0.1 M ammonium acetate and a linear conversion to 90% acetonitrile was tested, as proposed by Eerola et al. (1993). Two Chromolith columns were coupled, so the retention times of the BAs were prolonged and they could be separated from the more polar peaks. However, in order to separate the CAD/HIS and IS/SER pairs, the change of the mobile phase B composition was necessary. Therefore methanol as an organic modifier was added, and the original mobile phase B (100% acetonitrile) was replaced by a mixture composed of acetonitrile and methanol (75:25, v/v). This change improved the selectivity of both coeluting pairs of compounds. Furthermore, ammonium acetate was eliminated, as it exerted no visible positive effect on the reduction of the peak tailing. Thus, the 0.1 M ammonium acetate contribution to the mobile phase A was eventually replaced by ultra-pure water. To facilitate mixing of the two mobile phases at the consecutive gradient steps and to prevent microbial growth, mobile phase A was composed of 50% water and 50% organic solution. The gradient program was recalculated, so in the ultimate version it was starting from 100% (and not from 50%) of mobile phase A. The final gradient employed for the separation of dansylated biogenic amines is described in Section 2.6.

Because dabsylated BAs are more hydrophobic than the dansylated compounds, the gradient proposed for the separation of dansylated amines had to be slightly modified. For the elution of the most hydrophobic compounds (such as SPD and SPM) a faster increase in solvent strength was recommended. The gradient used for the separation of dabsylated biogenic amines is given in Section 2.6.

Although the total analysis time of the dabsylated amines is by 5 min longer than that for the dansylated compounds, this difference is repaid by the advantage of obtaining an interference-free chromatogram. In the case of dansylated amines, PUT could not be separated from the excess reagent and the impurities, and its quantification was unfeasible. In general, the derivatisation

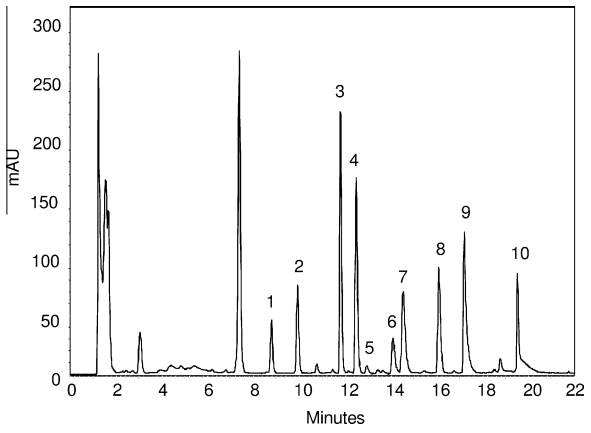

Fig. 5. Chromatogram of dabsylated amines extracted from a dry fermented sausage sample spiked at a concentration of 100 mg·kg^{-1} meat. Numbers as in Fig 1.

reagent type does not influence the elution order of the BAs, except for SER and 1,7-diaminoheptane (IS). In the case of dabsylation serotonin elutes just before the internal standard (Fig. 5), while in the case of dansylation it leaves the column after it.

3.4. Method validation

The newly elaborated method which includes dabsyl derivatisation and the optimised SPE purification underwent the validation procedure, in order to make it possible to use this method in the future routine analysis of food samples (such as dry fermented sausages).

3.4.1. Linearity and range

Standard calibration curves ($n = 7$) were built by plotting the relative responses (area ST/area IS) versus the concentration of each biogenic amine standard. For all standard curves, $R^2 \geqslant 0.998$ was observed. The instrumental detection limit was estimated as the concentration of the standard solution at the level of the signal-to-noise ratio equal to three (LOD: S/N = 3). For PHE, PUT, CAD, TYR, SPD and SPM, the LOD level was lower than 0.008 μg·mL^{-1} and for TRYP, SER and HIS, the LOD level was ca. 0.040 μg·mL^{-1}.

3.4.2. Matrix effect

To check the matrix effect, a calibration curve in the meat matrix was elaborated. Therefore the fermented meat samples were spiked with working solutions of the biogenic amine standards at the concentration levels of 1, 2, 5, 10, 25, 50, 75 and 100 mg·kg^{-1} meat. A *t*-test was performed to confirm the equality of the slopes of the standard calibration curve and the calibration curve developed in the meat matrix (Loco, 2006). As $t(b) \leqslant t^{\alpha=0.025}_{n_s+n_k-4}$, the slopes of the calibration curves in the meat matrix did not significantly differ from those obtained for the standard calibration curves. Thus, it can be concluded that the standard calibration curves can be used for quantification of the target compounds in the meat samples.

3.4.3. Method detection limit and quantification limit

The method detection limit (MDL) was estimated by spiking eight dry fermented meat samples at a concentration of 1 mg·kg^{-1} meat. The samples were subjected to the full analytical procedure. Because no blank meat samples are available, the non-spiked meat samples were analysed as the blanks, in order to subtract the respective values from the data valid for the spiked samples. The MDL can be calculated as $t_{(n-1,1-\infty=0.99)} \times$ standard deviation, where the Student's *t*-value for the 8 samples at the 99% confidence level was 2.998. The limit of quantification (LOQ) was expressed as the concentration at which the measurement precision was still acceptable (10% RSD). The MDL and LOQ-values of the target compounds are given in Table 1.

3.4.4. Accuracy and precision

Dry fermented sausage samples ($n = 6$) were spiked at two levels, i.e. at the 50 and 100 mg·kg^{-1} meat level, in order to evaluate the accuracy (expressed as recovery) and precision (repeatability) of the analytical procedure. Relative standard deviation (RSD) was used to express the precision, and the recovery (T) was calculated. In Table 1, the recovery and repeatability data for all biogenic amines are given. The relative standard deviation (RSD) for each biogenic amine determined in dry fermented meat samples was lower than 5%.

4. Conclusion

In dry fermented meat analysis, the most commonly applied HPLC-based method to determine biogenic amines includes a dansylation step. However, this method suffers from selectivity problems due to the presence of an excess reagent and the impurity peaks. Therefore the use of highly irritating ammonia is unavoidable, but even this procedure cannot remove the interfering peak completely, which makes quantification of putrescine unfeasible. Contrary to that, the dabsylation can easily be stopped in an ice bath, without any use of ammonia or other scavenging reagent. Moreover, the derivatisation temperature in the dabsylation procedure can be raised to 70 °C, without any loss of the analytes. As a result, the reaction time can be reduced to 20 min instead of the 45-min period with dansylation running at 40 °C.

Table 1
Repeatability (*r*) expressed as RSD (%) and the recovery (*T*, mean ± standard deviation) for dry fermented sausage samples spiked at two levels (50 and 100 mg·kg^{-1} meat), the method detection limit (MDL, mg·kg^{-1} meat) and limit of quantification (LOQ, mg·kg^{-1} meat).

Dry fermented sausage		Addition level I: 50 mg·kg^{-1} meat			Addition level II: 100 mg·kg^{-1} meat			MDL	LOQ
	Initial content (mg·kg^{-1})	Content after addition (mg·kg^{-1})	RSD (%)	*T* (%)	Content after addition (mg·kg^{-1})	RSD (%)	*T* (%)		
TRYP	2.11	53.21	1.92	102.19 ± 2.23	99.81	1.58	97.70 ± 1.58	0.94	3.15
PHE	nd	44.24	2.31	88.49 ± 2.24	89.85	3.30	89.85 ± 2.96	0.15	0.35
PUT	0.86	47.29	3.26	92.86 ± 3.38	95.43	2.11	94.47 ± 2.01	0.15	0.39
CAD	0.58	48.06	2.28	94.95 ± 2.41	98.72	1.99	96.50 ± 1.94	0.10	0.32
HIS	nd	43.01	3.21	86.02 ± 3.35	88.45	3.81	88.45 ± 3.60	0.90	3.10
SER	nd	42.98	4.34	85.96 ± 4.09	87.20	3.08	87.20 ± 2.69	0.20	0.47
TYR	nd	48.25	3.98	96.50 ± 4.21	94.06	3.86	94.06 ± 3.63	0.96	3.21
SPD	1.07	47.54	3.53	92.95 ± 3.67	90.71	3.49	89.64 ± 3.17	0.20	0.65
SPM	1.54	49.83	3.14	96.57 ± 3.42	90.27	3.51	88.73 ± 3.17	0.88	2.92

To ensure the baseline separation, the feasibility of an extra purification step was explored. The use of a classical LLE method with diethyl ether as an extraction solvent proved insufficient. Instead, a C18-SPE clean-up step of the derivatised extract was devised, which resulted in a repeatable procedure. This procedure included an acid-founded (0.4 M $HClO_4$) washing step and an elution with an organic solvent (acetonitrile). The sensitivity was improved by the possibility to concentrate the sample in an absence of interfering compounds.

Thus in order to develop a robust method to separate and quantify biogenic amines (with the most sensitive histamine as a critical BA case), one has to dissolve the extract residue in the solvent mixture identical to the mobile phase A and to store the obtained solution under refrigeration until the time of analysis. Application of buffering solutions (9 mM NaH_2PO_4) to prevent crystallisation of the amines was discarded, since addition of the buffer negatively affected the stability of the natural polyamines SPD and SPM.

Finally, the alternative method combining dabsyl derivatisation and SPE purification successfully passed the validation procedure. It is confirmed that with the use of this method, the 9 investigated biogenic amines originating from a complex matrix such as dry fermented meat, can easily be identified and reliably quantified.

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