Dispersive liquid-liquid microextraction combined with gas chromatography-mass spectrometry for *in situ* determination of biogenic amines in meat: estimation of meat's freshness

Wojciech Wojnowski*, Jacek Namieśnik, Justyna Płotka-Wasylka

Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry

11/12 Gabriela Narutowicza Street, 80-233 Gdańsk, Poland

*wojciech.wojnowski@pg.edu.pl, +48583486411

Abstract

A dispersive liquid-liquid microextraction (DLLME) gas chromatography-mass spectrometry (GC-MS) technique was developed for the determination of selected biogenic amines (BAs) in samples of poultry, pork and beef. Prior to the extraction process, an appropriate volume of sodium hydroxide solution was added to each of the portioned samples. Next, samples were homogenized, centrifuged and finally sonicated at an increased temperature. After another centrifugation, the supernatant was made up to 50 mL in a calibrated flask. Subsequently, 5 mL of supernatant was separately subjected to a derivatization and extraction procedure. A mixture of methanol (dispersive solvent; 210 µL), chloroform (extractive solvent; 300 µL), and isobutyl chloroformate (derivatizing reagent; 100 µL) was used in the extraction process together with an admixture of pyridine and HCl in order to eliminate the by-products. The application of the method enables fast derivatization and extraction of the BAs and a straightforward and rapid sample enrichment. It displayed good linearity, intra- and inter-day precision and good recoveries. The proposed methodology is characterized by low limits of detection and quantification (0.003-0.009 µg/g and 0.009-0.029 µg/g, respectively). The green character of the method was established based on the results of two tools, namely the Analytical Eco-Scale and GAPI. It was successfully used to analyse samples of poultry, porcine and bovine meat. Multivariate statistical data analysis was applied in order to evaluate the potential use of the determined BAs as spoilage markers of particular meat types.

Keywords: biogenic amines; dispersive liquid-liquid microextraction; meat; shelf-life; gas chromatography-mass spectrometry

1. Introduction

The organoleptic qualities of fresh meat and poultry deteriorate during storage. However, sensory analysis is often not sufficient to detect early indications of spoilage, and so methods such as the total viable bacteria counts and the determination of the total volatile basic nitrogen (TVB-N) are used to assess the freshness of meat products [1]. In the latter, the content of ammonia produced during deamination of amino acids is linked to the progress of putrefaction [2]. An alternative approach to the assessment of meat and poultry freshness is the determination of biogenic amines (BAs), as they are formed from precursor amino acids through the enzymatic decarboxylation during storage [3]. The determination of BAs is suitable for detecting early onset of spoilage, as the ones naturally occurring in the animal tissues could be degraded by certain microorganisms [4,5]. Furthermore, apart from

being indicators of spoilage, BAs themselves can have a detrimental effect on human health when ingested. Histamine (HIST) has been linked to several outbreaks of food poisoning, while tyramine (TYR) is associated with the hypertensive crisis. The toxicity of HIST is compounded by the presence of cadaverine (CAD), putrescine (PUT) and TYR, and since BAs are the precursors of nitrosamines they should also be considered as potential carcinogens [6].

Because of the complexity of the matrix and the nature of amino acids decarboxylation due to microbial enzymes and tissue activity, the concentration of a single BA might not be a sufficient marker of spoilage. For this reason, several meat freshness indices have been proposed. In particular, the Chemical Quality Index (CQI) is the sum of concentrations of CAD, PUT, spermine (SPER), spermidine (SPERM) and HIST [7], and the Biogenic Amines Index (BAI) is the sum of concentrations of HIST, CAD, TYR and PUT [8]. Silva *et al.* have also proposed a chicken meat quality index based on the ratio of SPERM and SPER [9]. However, the reliability of these indices in detecting the early stages of putrefaction relies greatly on the capabilities of the analytical method used for the determination of BAs.

The techniques used for the determination of biogenic amines in poultry and meat samples include ion chromatography, capillary electrophoresis, gas chromatography (GC) and high-performance liquid chromatography (HPLC), with the latter being the most popular [10,11]. However, the application of HPLC is often relatively laborious and entails the use of relatively large volumes of organic solvents [12]. On the other hand, the direct determination of BAs in meat samples using GC is difficult due to their relatively low concentration and interferences from e.g. polyphenols [13]. These shortcomings can be alleviated using extraction and derivatization which increases the amines' volatility and facilitates detection using GC. In particular, dispersive liquid-liquid microextraction (DLLME) is relatively inexpensive, easy to perform, rapid and characterised by high enrichment factor and recovery. Moreover, as it requires the use of only small volumes of solvents it conforms to the postulates of green analytical chemistry [14]. DLLME-GC-MS has previously been used for the determination of polycyclic aromatic hydrocarbons in grilled meat [15] and of BAs in food samples [16–19]. However, to the authors' best knowledge a dedicated method involving the use of this technique for the determination of BAs in animal tissues has not yet been described.

The aim of this study was to develop and validate a DLLME-GC-MS analytical method for the determination of BAs in meat samples for the purpose of freshness assessment, especially in the context of meat freshness indices. These indices are increasingly being used as a quantitative method to evaluate the shelf-life of fresh meat, and so rapid and reproducible methods for the determination of BAs in this matrix might find immediate application. Particular focus has been placed on the sample preparation procedure. Since the meat samples are solid, additional steps were introduced prior to the extraction stage. Thus, an appropriate volume of sodium hydroxide solution was added to each of the portioned samples. Next, samples were homogenized, centrifuged and finally sonicated at an increased temperature. After another centrifugation, the supernatant was made up to 50 mL in a calibrated flask. Then, 5 mL of supernatant was separately subjected to a derivatization and extraction procedure. In the extraction stage, a mixture of methanol (dispersive solvent; 210 µL), chloroform (extractive solvent; 300 µL), and isobutyl chloroformate (derivatizing reagent; 100 µL) were used in the extraction process together with an admixture of pyridine and HCl in order to eliminate the by-products. Care has been taken to evaluate the impact of the nature and amount of both the derivatizing reagent and the extractive and dispersive solvents, as well as the reaction time. The

developed method was used to determine the concentration of selected BAs in the samples of fresh chicken, pork and beef during storage in different containers. Multivariate statistical data analysis was used to determine the applicability of these BAs as meat freshness indicators.

2. Experimental

2.1. Materials and reagents

The biogenic amine standards: CAD (≥99.0%), dimethylamine (DIMET, 99%), HIST (≥99.0%), PUT (≥99.0%), SPER (≥99.0%), tryptamine (TRP, 99%), TYR (≥98%) and 2-phenylethylamine (2-PE, ≥98%) were obtained, mostly in the form of hydrochloride salts, from Sigma Aldrich (Steinheim, Germany), as was the internal standard (hexylamine, IS). The derivatizing reagents ethyl chloroformate (ECF) and isobutyl chloroformate (IBCF) were also supplied by Sigma Aldrich. High purity grade dispersive solvents acetone and methanol (MeOH) were obtained from Fluka (Buchs, Switzerland). The extractive solvents isooctane, chloroform and dichloromethane of high purity HPLC analysis grade were obtained from Sigma Aldrich. 5 M HCl was obtained from Fluka. Other chemicals were of analytical grade. The solution of alkaline methanol was prepared by dissolving KOH in methanol until saturation. The silanized screw-capped vials with solid PTFE-lined caps were obtained from Supelco (Bellefonte, PA, USA). The manual homogenizer (Bamix ESGE Ltd., Mettlen, Switzerland) at 14.000 rpm was used for homogenization. Centrifuge (Combi–Spin FVL-2400N, Biossan, Latvia) was used for centrifugation performed at 4 °C and 5000 rpm for 15 min. Bandelin SONOREX (Sigma Aldrich, Steinheim, Germany) was used for ultrasonication.

2.2. Sampling

Samples of fresh chicken breast muscle (*pectoralis major*, 1C-5C), pork loin (*longissimus dorsi*, 1P-5P) and beef loin (*longissimus dorsi*, 1B-5B), five each, were obtained from a local distribution centre in Gdańsk, Poland. Each sample weighed 100 g. All samples were immediately refrigerated and transported in a portable cooler to the laboratory within 30 min, where they were stored at 4°C in three different containers: in a aerobically in a standard PP-R food box (I), polypropylene co-polymer (PP-R) vacuum food box (II), and aerobically in a standard high-density polyethylene (HDPE) refrigerator bag (III). All samples were from adult animals, and pieces were taken for analyses 1, 3, and 5 days post-mortem. Each sample was analysed in triplicate.

2.3. Preparation of standards solution

Stock solutions (1mg/mL) of BAs were prepared by weighing each analyte standard and dissolving in 10 mL of deionized water. A multi-compound working standard solution (1 µg/mL) of each compound was prepared by appropriate dilution. The solutions were stored at 4 °C in silanized screw-capped vials with solid PTFE-lined caps. All calibration and working solutions were prepared by sequentially diluting the stock solutions in an appropriate linear range with a spiked IS on the day of the analysis. The IS solution was prepared at 1 mg/mL and diluted to 0.1 µg/mL with deionized water during sample analysis.

2.4. Preparation of samples

The sample preparation procedure was the same for each kind of meat. The same amount of meat samples (5 g) was added to 50 mL of 0.1 M NaOH, homogenized using a laboratory mixer and centrifuged for 15 min at 4 °C and 5000 rpm. Samples were then placed in a PTFE vessel and placed for

60 min in an ultrasonic bath thermostated at 70 °C. The homogenised mixture was centrifuged at 5000 rpm for 3 min, the supernatant was collected and subsequently made up to 50 mL in a calibrated flask. Three aliquots of supernatant (5 mL each) were separately subjected to a derivatization and extraction procedure.

2.5. Derivatization and dispersive liquid-liquid extraction methodology

For the in-situ derivatization coupled to DLLME, an aliquot of 5 mL of the extract obtained during the previous step was spiked with an internal standard (50 µL of a water solution containing the internal standard) and placed in a glass centrifuge tube with conical bottom containing 0.5 g NaCl. Next, a 5 M HCl solution was added to obtain pH 11. A mixture of methanol (600 µL), pyridine:HCl (100 µL, 1:1 v/v) and isobutyl chloroformate (200 µL) was rapidly injected into the sample tube, and the mixture was again gently shaken for a few seconds. After 10 min, a 1 mL of chloroform was added and after centrifugation for 5 min at 5000 rpm, the extraction solvent was sedimented in the bottom of the conical tube. The bottom layer was transferred to vials with 100 µL inserts. A 5 µL aliquot was injected in the splitless mode into the GC–MS system.

The relative response factors (RRFs) was used to express the effectiveness of extraction as well as derivatization procedure and were calculated according to the following equation (1):

$$RRF = \frac{A_S \times C_{Is}}{A_{IS} \times C_s} \qquad (1)$$

AIS: the internal standard peak area,
CS: the target analyte concentration (g/mL),
AS: the target analyte peak area,
CIS: the internal standard concentration (g/mL).

2.6. Gas chromatography-mass spectrometry method

The gas chromatograph 7890A (Agilent Technologies, Santa Clara, CA, USA) equipped with an electronically controlled split/splitless injection port was interfaced with a mass selective detector (5975C, Agilent Technologies, Santa Clara, CA, USA) with EI ionization chamber. GC separation was performed on Zebron ZB-5MS capillary column (30 m x 0.25 mm I.D., 0.25 µm film thickness) (Phenomenex, Torrance, CA, USA). The injection was made in splitless mode (injection pressure 32 ps) at 240 °C. Helium was the carrier gas with a constant pressure of 30 psi. The oven temperature program was as follows: 45 °C held for 2 min, ramped to 160 °C at 15 °C/min and held for 2 min, and ramped to 280 °C at 10 °C/min and held 9 min. The total run time was 33 min. The MS transfer line temperature was held at 280 °C. Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy; ion source temperature, 250 °C. The MS system was routinely set in SIM mode and each analyte was quantified based on peak area using one target and one or more qualifier ion(s) (Table 1). Agilent ChemStation software was used for data collection and GC-MS control.

Table 1. Fragments, relative intensities and retention time (Rt) of BAs obtained by application of GC-MS technique.

Analytes	m/z SIM ions (Relative intensities)	Rt

DIMET	**72 (90)**	90 (99)	145 (2)			2.02
Hexylamine (IS)	**146 (99)**	130 (77)	128 (15)			8.12
SPER	**101 (80)**	144 (99)	201 (32)	274 (4)		8.51
2-PE	**130 (99)**	104 (80)	91 (76)	221 (31)	148 (19)	9.99
PUT	**170 (99)**	130 (64)	288 (11)			12.00
TRYP	**130 (99)**	143 (59)	260 (19)	187 (4)		13.00
TYR	**120 (99)**	107 (29)	176 (5)	237 (2)	337 (1)	13.51
CAD	**130 (79)**	84 (82)	129 (74)	302 (3)		13.71
HIST	**194 (99)**	238 (17)	138 (26)			14.32

159

160 2.7. Quality assurance

161 Matrix effects (ME) were investigated at two concentration levels, 0.5 and 5 µg/L and were calculated
162 by comparing the responses (peak area of each analyte against peak area of the IS) for appropriate
163 solution of analytes prepared in methanol (sets A, n=3) with those measured in blank meat extracts
164 spiked after the extraction procedure with the same amount of analyte (sets B, n=3). The following
165 formula was used (2):

166 $$ME[\%] = \frac{B}{A} \times 100\% \qquad (2)$$

167 The optimized method was validated for linearity, detection and quantification limits (LOD and LOQ,
168 respectively), selectivity, accuracy and precision. The method's linearity was investigated by a
169 regression analysis of the relative area versus the analyte concentration. The relative area was
170 presented as the ratio between the peak area of a particular BA and the peak area of the IS. The LODs
171 were calculated as three times the signal-to-noise ratio, while LOQ were calculated as ten times the
172 signal-to-noise ratio. The intra-day precision was investigated by analysing four replicates of meat
173 samples spiked at 0.5 µg/L on the same day. Inter-day precision was investigated by means of samples
174 analysis on two different days over a period of two weeks. The recovery was calculated by comparing
175 unspiked extract samples to ones spiked at 0.5 µg/L; n=4.

176 2.8. Evaluation of the green profile

177 The developed analytical procedures used for the determination of biogenic amines in meat samples
178 were subsequently assessed in terms of 'greenness' by two well-established methods: the Analytical
179 Eco-Scale and the Green Analytical Procedure Index (GAPI).

180 2.9. Multivariate statistical analysis

181 The determined concentration values of BAs in meat samples were used as input data for multivariate
182 statistical data analysis using a dedicated Python toolkit Orange v.3.13 [20]. Initial data processing
183 involved standardization (centring by the mean value and scaling by standard deviation). The analysis
184 of variance within the variables and feature selection was performed using the ReliefF algorithm [21],
185 as it is more sensitive to feature interactions, especially with discrete features (e.g. days of storage,
186 packaging material) compared to ANOVA or chi^2 [22]. The area under the ROC curve, classification

accuracy and precision of supervised classification (naïve Bayes) was validated using a 10-fold stratified cross-validation. Missing data (determined concentration below LOQ) was replaced by the value LOD/3. Hierarchical cluster analysis with Ward linkage was performed based on Mahalanobis distances. Height ratio of 66% was assumed for the identification of relevant clusters.

3. Results and discussion

3.1. Optimization of extraction conditions

In the DLLME procedure coupled with derivatization process, the fundamental parameters that need to be optimized are the extractive and dispersive solvents, solvents volume, type and volume of the derivatization reagent, and the extraction and derivatization time. These parameters were systematically studied in order to achieve a good sensitivity, selectivity and precision for all BAs determined in the study.

3.1.1. Selection of extractive, dispersive solvents and of the derivatizing agent

For the extractive solvent selection, following requirements were considered: immiscibility with water, density in relation to water, high extraction capability, compatibility with the derivatizing reagent, good solubility of derivatives, and good chromatographic behaviour. Based on these criteria the three following solvents were examined: isooctane (density: 0.83 g/mL), dichloromethane (density: 1.33 g/mL) and chloroform (density: 1.48 g/mL). For the selection of dispersive solvent, the miscibility of the dispersive solvent in the extractive solvent as well as in the sample solution were the features taken into account. Two solvents: acetone and methanol (MeOH) were examined.
In this study, the group of chloroformates were examined as potential derivatizing agents. It is reported that alkyl chloroformates are a group of derivatizing reagents with very favourable characteristics in regard to the determination of BAs using the GC technique. In addition, these derivatizing agents do not require specific condition during derivatization step which can be performed in a short time. Moreover, they are cheap, commercially available and simple to use. In the present study, two derivatization reagents belonging to this group, namely ethyl chloroformate (ECF) and isobutyl chloroformate (IBCF) were examined.

For this experiment, extractions were carried out for 15 min from 5 mL of supernatant of meat sample (with pH adjusted to 11) spiked with all the BAs and 100 µL of derivatizing reagent with a combination of 300 µL of MeOH or acetone, 100 µL of mixture of pyridine and HCl (1:1; in order to omit the by-products). After 5 minutes 300 µL of isooctane, dichloromethane or chloroform were added to the solution. The obtained results are listed in Table 2.

220 Table 2. Information on peak area obtained by GC-MS for analytes of interest by using different method conditions

221

Analyte	Extractive solvent											
	Dichloromethane				*Chloroform*				*Isooctane*			
	Dispersive solvent											
	MeOH		*Acetone*		*MeOH*		*Acetone*		*MeOH*		*Acetone*	
	Derivatizing reagent											
	IBCF	*ECF*	*IBCF*	*ECF*	*IBCF*	*ECF*	*IBCF*	*ECF*	*IBCF*	*ECF*	*IBCF*	*ECF*
CAD	10053	91124	6745	5683	50006	37234	7142	5987	n.d.	n.d.	n.d.	n.d.
DIMET	105432	109279	4647	n.d.	56675434	51623712	5134	4782	n.d.	n.d.	9102	7893
HIST	n.d.	n.d.	3829	2034	100118	72312	4345	3123	n.d.	n.d.	n.d.	n.d.
PUT	153078	200542	10734	8965	404298	312941	11765	9165	n.d.	n.d.	n.d.	n.d.
SPER	702000	598424	98356	25785	4154005	3334012	100351	41783	n.d.	n.d.	n.d.	n.d.
TRP	385439	219654	10429	10525	1010300	993912	45329	21052	n.d.	n.d.	n.d.	n.d.
TYR	n.d.	n.d.	n.d.	n.d.	267309	200081	5643	4321	n.d.	n.d.	n.d.	n.d.
2-PE	1667432	1300976	678954	457042	8166501	6204192	704952	500040	n.d.	n.d.	100012	18290
IS	997532	975309	27123	11115	3575765	1990998	31098	20843	n.d.	n.d.	8992	7827

CAD, cadaverine; DIMET, dimethylamine; HIST, histamine; PUT, putrescine; SPER, spermine; TRP, tryptamine; TYR, tyramine; 2-PE, 2-phenylethylamine; IS, hexylamine; MeOH, methanol; N.D., not detected

222

Based on the results of the analysis it can be observed that the GC-MS responses to the analytes differed significantly from the responses to the solvents. Most of the derivative compounds were not extracted by isooctane, except for DIMET and 2-PE. Both dichloromethane and chloromethane could be successfully used as extractive solvents in the discussed scenario, however, the best extraction results were obtained when chloroform was used as extraction solvent. The extraction efficiency for most of the derivatives was higher when methanol was used compared to acetone. Both IBCF and ECF were used with satisfactory results, with all standards being detected, however, the use of IBCF has led to a higher peak response for all derivatives (Table 2), therefore, only this reagent was tested in the further study. To the authors' best knowledge, no derivatization study for BAs in poultry and meat samples using IBCF (and other alkyl chloroformates) has yet been published. Thus, different volumes of this compound (50 µL, 80 µL, 110 µL, 140 µL) were admixed during 16 experiments carried out at room temperature for 5, 10, 15, and 20 min (Table 3: experiments 1A–4D). The use of the coupling of chloroform and methanol gave the best results and they were chosen as the extraction and disperser solvents, respectively for the following experiments. Based on the above information, the following reagents were used in further studies: MeOH, chloroform and IBCF.

3.1.2.Optimization of the volume of dispersive and extractive solvents

In order to assess the impact of the extractive solvent volume on the efficiency of the extraction, a constant volume of dispersive solvent (MeOH, 300 µL), as well as the constant volume of pyridine and HCl mixture (100 µL, 1:1 v/v), was subjected to the same procedure. IBCF was used as a derivatizing reagent (100 µL). Different volumes of chloroform (from 100 µL to 500 µL) were examined. Due to the fact that the volume of the upper phase was low in case of an admixture of 100 µL and 200 µL of chloroform, there were issues with reproducibility (replicates were impracticable). However, the volume of the upper phase increased when a higher volume of extractive solvent was used (300, 400 and 500 µL). The enrichment factors were calculated using the following equation:

$$Enrichment\ factor = \frac{\%Recovery \times \frac{V_{aq}}{V_{sed}}}{100} \qquad (3)$$

(V_{aq}- the volume of the aqueous phase, V_{sed}- the volume of the sedimented phase)

The enrichment factor decreased significantly with the increase of the volume of extracting solvent (Figure 1). Thus, 300 µL of chloroform was selected in order to obtain high enrichment factors and low detection limits.

In order to assess the impact of the dispersive solvent volume on the extraction efficiency, different volumes of MeOH (150 µL, 180 µL, 210 µL, 240 µL, 270 µL) containing 100 µL of IBCF and a fixed volume of pyridine and HCl mixture (100 µL; 1:1 v/v) were examined. The results indicated that with the increase of dispersive solvent volume the extraction efficiency was higher (150 to 210 µL), and then slightly decreased (210, 240, 270 µL) for all derivatives. Thus, based on experimental results 210 µL of MeOH was chosen as the optimum volume for the dispersive solvent. Influence of the volume of methanol on the peak area of BAs by DLLME–GC–MS is shown in Figure 2.

3.1.3.*Selection of the volume of derivatizing agent and reaction time*

The concentrations of the target compounds as well as IS (0.5 µg/L) used in each experiment were constant. The conditions of GC-MS measurement applied during the examination of the impact of derivatising conditions on the yield of derivatised target compounds were also the same. RRFs were calculated for the analytes in order to assess the effectiveness of derivatization performed under the different reaction conditions.

Table 3**.** Different conditions of the derivatization process used for the chemical conversion of the target compounds by DLLME-GC-MS

Experiment no.	Volume of DR [µL]	Reaction time [min]
1A	50	5
2A		10
3A		15
4A		20
1B	80	5
2B		10
3B		15
4B		20
1C	110	5
2C		10
3C		15
4C		20
1D	140	5
2D		10
3D		15
4D		20

Due to the fact that the internal standard is not subjected to derivatization, a higher value of RRFs indicated an increase in reaction effectiveness. This knowledge was used to compare the effectiveness of the derivatization processes carried out at different reaction conditions. Information on the calculated RRFs (as mean value, *n* = 3) calculated based on the GC-MS results of experiments of 1A–4D for the target compounds are listed in Table 4. The relative standard deviations (RSD) of all RRFs were <3.3%.

Table 4. Information on RRFs (mean value; *n* = 3; RSD < 3.3%) calculated from the obtained GC-MS results for derivatives of analytes under the chromatographic conditions of experiments 1A–4D, as shown in Table 3.

DR	IBCF															
Experiment	1A	2A	3A	4A	1B	2B	3B	4B	1C	2C	3C	4C	1D	2D	3D	4D
Analyte	RRF parameters (mean value) (n=3) [x10^{-3}]															
CAD	n.d.	99	134	152	n.d.	102	216	201	n.d.	117	200	187	n.d.	111	199	156
DIMET	115	158	352	509	299	715	1009	911	329	732	917	852	317	672	897	809
HIST	n.d.	172	201	157	101	167	300	243	n.d.	160	272	207	145	142	237	157
PUT	n.d.	143	181	132	n.d.	145	312	293	n.d.	161	291	265	ND	118	263	178
SPER	58	300	414	456	201	506	802	762	199	511	776	748	186	432	743	604
TRP	n.d.	113	331	298	n.d.	276	423	408	n.d.	251	401	382	n.d.	201	378	278
TYR	89	301	519	406	269	645	834	776	201	621	800	678	189	598	748	654
2-PE	n.d.	101	322	218	n.d.	249	421	356	n.d.	219	377	309	n.d.	200	332	265

Based on the calculated RRFs it can be concluded that the derivatization process with IBCF depends strongly on the time parameter as well as on temperature. The efficiency of target compound derivatization with appropriate conditions: 80 µL of IBCF for 15 min (3B) was the highest and thus, these reaction conditions were selected as the optimum for further study.

3.2. Results of quality assurance

No statistically significant differences were observed (P>0.1) during the examination of the matrix effect, and so quantification was performed by internal calibration. The values of correlation coefficients (R) were good (R > 0.996) demonstrating excellent linearity for the studied range. The LODs ranged from 0.003 to 0.009 µg/L and the LOQs ranged from 0.0099 to 0.029 µg/g. Information regarding these parameters is listed in Table 5. The relative standard deviation for intra-day precision ranged from 2% to 5%, while the RSD for inter-day precision ranged from 3% to 6%. The EFs were calculated as shown in Equation 2, and values between 32 and 48 were attained. The values of average recovery ranged from 79 to 101 % as can be seen in Table 5. The experiment was not carried out beyond five days of storage, since at this point the changes of the meat's properties can already be detected using sensory analysis, especially in the case of poultry [23].

Table 5. Information on linearity, average recoveries (%), intra-day and inter-day repeatability (%RSD), limits of detection and limits of quantification obtained with the optimized method in spiked samples, analyzed by GC-MS (*n* = 4 at each level).

Analyte	Linearity (µg/L)	R	Concentration level 0.5 µg/L		Inter-day (%RSD)	LOD (µg/g)	LOQ (µg/g)	EF
			Recovery (%)	Intra-day (%RSD)				
CAD	0.05-10 10-500	0.997	97	4	5	0.003	0.0099	32
DIMET	0.05-10 10-500	0.996	96	5	5	0.004	0.013	48
HIST	0.05-10	0.998	98	2	3	0.006	0.019	42
PUT	0.05-10	0.998	101	3	4	0.005	0.017	38
SPER	0.05-10	0.997	79	4	5	0.009	0.029	33
TRP	0.05-510	0.996	81	3	6	0.007	0.023	35
TYR	0.05-10	0.998	87	4	3	0.007	0.023	42
2-PE	0.05-10	0.996	93	5	5	0.004	0.013	46

LOD, LOQ calculated with respect to the weight of the respective solid matrix

3.3. Assessment of the noxious impact on the environment using Analytical Eco-Scale and GAPI

The concept of Green Analytical Chemistry (GAC) has been introduced to analytical practice due to concerns connected with a sustainable environment which resulted in a focus being placed on reducing or completely eliminating the use of solvents and other chemicals which are toxic and hazardous. In this context, eco-friendly as well as clean practices have been implemented in different fields of research. As was mentioned previously, the BAs determination in meat samples is mainly carried out using HPLC after extraction (mainly liquid-liquid extraction) and chemical conversion of analytes, which are not considered 'green'. The procedure described in this study is based on a micro-scale extraction technique and GC-MS. To evaluate its 'green' character, the Analytical Eco-Scale and GAPI tools were applied. In addition, the developed procedure was compared to one based on the ultra-performance liquid chromatography (UHPC) technique for final determination.

The Eco-Scale tool is a semi-quantitative tool, based on assigning penalty points (PPs) to parameters of an analytical process that are not in agreement with an ideal green analysis. It is simple and fast to perform and has well-defined criteria of evaluation. For each analytical protocol, PPs are given if it deviates from desired green parameters which are quantitatively connected to following factors: reagents amount and its hazards, waste production and energy consumption. The fundamental concept of the analytical Eco-Scale is that the ideal green analysis has a value of 100, thus, the closer to the highest score, the greener the procedure [24]. The sum of PPs for the whole evaluated procedure is subtracted from the ideal score of 100 to obtain the Eco-Scale score. The concept of the Analytical Eco-Scale assumes that the score of ≥75 represents an excellent green analysis, ≥50 represents an acceptable green analysis, and <50 represents inadequate green analysis. Thus, considering PPS given for the described procedure (25 PPs) it can be assumed that it represents a green analysis. The same cannot be said about the reported procedure based on UHPC, where the sum of PPs for the entire methodology is 36 which means that the protocol is merely acceptable in terms of 'greenness'. The results of this assessment were confirmed based on the analysis of GAPI pictograms (Figure 3). This index is a 'green' assessment tool of analytical protocols which rates analytical methods against the amount and type of waste, environmental hazard and chemical health, and energy requirements [25]. This tool presents in a pictorial form information on the entire analytical protocol, from sampling, through sample preparation to a final determination.

3.4. Analysis of real samples

The results of the determination of BAs in samples of pork, beef and poultry are listed in Tables 6-8. These are average values of the results of analysis of five separate samples, each performed in triplicate. They are in agreement with previously reported values [2,5,26,27]. There are noticeable differences between the content of BAs in samples stored in different packaging materials, however, the variance is mostly due to the duration of refrigerated storage. The result of PCA is shown in Figure 5. Based on the plot of the two first principal components it can be observed that storing samples in vacuum containers does not produce effects as evident as in the case of modified atmosphere packaging (MAP) [28], although the differences do become more pronounced over time. Based on the cluster analysis (Figure 4), it can be noticed that the BAs can be grouped into three distinct clusters based on the distances between data points in a multi-dimensional space. If only several were to be selected for a meat quality index, they should not be limited to the ones grouped within a single cluster, as this would likely limit the performance of the model.

The impact of packaging was the greatest in the case of TYR for poultry (AUC 0.720), DIMET and SPER for pork (AUC 0.713) and HIST and SPER for beef (AUC 0.642).

In the case of poultry, the four amines which displayed the greatest variance in the terms of storage time were (in decreasing order) CAD, HIST, TYR and PUT which validates the applicability of the BAI index proposed by Veciana-Nogués et al. [29] in chicken meat freshness evaluation. However, perfect classification (AUC 1.000, CA 1.000, precision 1.000) was achieved when using only the concentration values of CAD and HIST as inputs, and the use of CAD alone allowed to obtain a good classification (AUC 0.996, CA 0.903, precision 0.917) which also supports earlier findings [30,31]. It has been suggested that the more rapid increase of the concentration of BAs in poultry meat as compared to

pork or beef can be attributed to the presence of shorter protein chains which facilitates the generation of amino acid precursors for their biosynthesis by proteolytic enzymes [30].

In pork, the greatest variance during storage was due to the changes in the concentration of CAD, HIST, TYR and 2-PE. All four had to be used as inputs to achieve perfect classification, however, good results were obtained when the amines of the BAI index were considered (AUC 1.000, CA 0.911, precision 0.930).

Finally, in the case of bovine meat, where the concentration of BAs increased, e.g. poultry, the four best-ranked BAs in terms of variance caused by the duration of storage were CAD, 2-PE, PUT and TRP which allowed for a good classification of samples (AUC 0.967, CA 0.889, precision 0.917).

Based on the results of the multivariate statistical analysis it can be assessed that for a general BA-based meat quality index, regardless of the type of sample, the most relevant amines are (in order of decreasing relevance) TRP, CAD, 2-PE and PUT, collectively allowing for a very good classification based on the duration of storage (AUC 0.994, CA 0.941, precision 0.941). A FreeViz projection (linear projection of multivariate data that best separates the instances of a different class [32]) of the entire data set is depicted in Figure 6.

Table 6. The concentration of BAs in samples of fresh chicken meat (mg/kg, average±MSE, *n*=5) refrigerated at 4 °C over a period of 5 days in 3 different containers: PP-R food box (I), PP-R vacuum box (II) and an HDPE bag (III)

BA	Container	Day of storage		
		1	3	5
2-PE	I	n.d.	n.d.	0.4506±0.0034
	II	n.d.	n.d.	0.3124±0.0014
	III	n.d.	n.d.	0.5950±0.0015
CAD	I	n.d.	8.706±0.044	10.414±0.048
	II	n.d.	7.818±0.012	9.806±0.047
	III	n.d.	9.150±0.019	11.042±0.032
DIMET	I	0.4828±0.0022	0.4166±0.0030	0.3654±0.0044
	II	0.4840±0.0029	0.4490±0.0052	0.3694±0.0031
	III	0.48140±0.00051	0.3946±0.0021	0.313±0.013
HIST	I	1.4814±0.0046	4.332±0.032	3.806±0.047
	II	1.4800±0.0047	4.114±0.030	3.654±0.037
	III	1.48460±0.00051	5.078±0.048	3.380±0.019
PUT	I	0.9884±0.0019	1.1160±0.0017	1.7958±0.0058
	II	0.9886±0.0018	1.033±0.017	1.5378±0.0052
	III	0.99140±0.00040	1.1498±0.0020	1.977±0.014
SPER	I	14.64±0.14	15.76±0.21	20.56±0.30
	II	14.58±0.14	14.860±0.068	19.620±0.058
	III	14.8±0	16.400±0.084	22.900±0.055
TRP	I	3.044±0.020	2.132±0.014	1.802±0.017
	II	3.038±0.018	2.552±0.030	1.914±0.015
	III	3.0440±0.0075	2.100±0.044	1.184±0.024
TYR	I	n.d.	3.116±0.012	4.100±0.020

	II	n.d.	2.418±0.015	3.050±0.038
	III	n.d.	4.130±0.011	5.314±0.036

366 Table 7. The concentration of BAs in samples of fresh pork (mg/kg, average±MSE, *n*=5) refrigerated at
367 4 °C over a period of 5 days in 3 different containers: PP-R food box (I), PP-R vacuum box (II) and an
368 HDPE bag (III)

BA	Container	Day of storage		
		1	3	5
2-PE	I	n.d.	n.d.	0.8034±0.0087
	II	n.d.	n.d.	0.495±0.017
	III	n.d.	n.d.	0.8502±0.0017
CAD	I	n.d.	6.330±0.059	8.73±0.11
	II	n.d.	4.870±0.061	7.202±0.033
	III	n.d.	6.768±0.029	9.16±0.11
DIMET	I	0.7790±0.0035	1.328±0.079	3.172±0.090
	II	0.7796±0.0032	1.028±0.024	2.166±0.031
	III	0.7792±0.0036	1.488±0.050	3.806±0.043
HIST	I	1.172±0.027	3.752±0.064	3.786±0.051
	II	1.180±0.028	3.250±0.039	3.390±0.019
	III	1.168±0.031	4.016±0.065	4.056±0.024
PUT	I	n.d.	0.762±0.014	2.254±0.079
	II	n.d.	0.6648±0.0033	1.616±0.035
	III	n.d.	0.9160±0.0021	2.628±0.047
SPER	I	12.34±0.15	10.180±0.086	8.676±0.050
	II	12.40±0.16	9.820±0.073	9.480±0.037
	III	12.38±0.17	9.340±0.040	7.180±0.022
TRP	I	3.346±0.025	4.42±0.11	2.250±0.033
	II	3.330±0.024	3.960±0.053	2.684±0.046
	III	3.312±0.024	4.720±0.062	2.134±0.039
TYR	I	0.2294±0.0027	1.118±0.031	3.280±0.057
	II	0.2328±0.0012	0.9726±0.0026	2.584±0.052
	III	0.2330±0.0021	1.354±0.028	3.406±0.036

369 Table 8. The concentration of BAs in samples of fresh beef (mg/kg, average±MSE, *n*=5) refrigerated at
370 4 °C over a period of 5 days in 3 different containers: PP-R food box (I), PP-R vacuum box (II) and an
371 HDPE bag (III)

BA	Container	Day of storage		
		1	3	5
2-PE	I	n.d.	n.d.	0.2222±0.0078
	II	n.d.	n.d.	0.2134±0.0025
	III	n.d.	n.d.	0.2448±0.0070
CAD	I	n.d.	n.d.	3.468±0.047
	II	n.d.	n.d.	3.228±0.052
	III	n.d.	n.d.	3.684±0.091
DIMET	I	0.61220±0.00020	0.7448±0.0052	0.9808±0.0087

	II	0.61240±0.00024	0.6812±0.0046	0.7880±0.0027
	III	0.61220±0.00020	0.7710±0.0064	1.115±0.040
	I	1.0034±0.0016	1.266±0.046	1.490±0.052
HIST	II	1.0042±0.0015	1.1132±0.0047	1.2156±0.0026
	III	1.0066±0.0016	1.396±0.023	1.630±0.040
	I	n.d.	n.d.	1.522±0.083
PUT	II	n.d.	n.d.	1.096±0.027
	III	n.d.	n.d.	1.716±0.067
	I	27.700±0.063	23.30±0.18	20.68±0.10
SPER	II	27.660±0.060	25.620±0.092	23.500±0.063
	III	27.580±0.058	21.12±0.10	16.82±0.53
	I	6.1220±0.0074	7.582±0.065	8.456±0.047
TRP	II	6.1280±0.0074	7.072±0.029	8.182±0.050
	III	6.1340±0.0060	7.844±0.021	8.814±0.043
	I	0.13080±0.00058	0.2086±0.0065	0.2372±0.0034
TYR	II	0.13100±0.00055	0.1636±0.0025	0.34±0.15
	III	0.13200±0.00063	0.2132±0.0076	0.2572±0.0012

4. Conclusions

The use of the DLLME GC-MS method allows for a relatively simple, rapid and simultaneous determination of BAs in meat products. The efficiency of the procedure for the extraction of BAs from complex meat matrices was confirmed by both, the obtained recovery values and the results of the real samples analysis. The extraction procedure was efficient and highly reproducible. The validation results, namely linearity, recovery, precision and limits of quantification and detection were very satisfactory. The low quantitation limits facilitate the use of BAs concentration values as meat freshness indicators at the early stages of spoilage, before the exponential increase of the concentration of the bacterial metabolites. It can be concluded that the developed procedure is suitable for rapid, reliable and inexpensive determination of BAs in fresh meat samples. Furthermore, it was assessed that tryptamine, cadaverine, 2-phenylethylamine and putrescine should be considered as potential meat freshness indicators when developing freshness indices based on the concentration of BAs.

References

[1] W. Wojnowski, T. Majchrzak, T. Dymerski, J. Gębicki, J. Namieśnik, Electronic noses: Powerful tools in meat quality assessment, Meat Sci. 131 (2017) 119–131. doi:10.1016/j.meatsci.2017.04.240.

[2] J.S. Min, S.O. Lee, A. Jang, C. Jo, C.S. Park, M. Lee, Relationship between the concentration of biogenic amines and volatile basic nitrogen in fresh beef, pork, and chicken meat, Asian-Australasian J. Anim. Sci. 20 (2007) 1278–1284. doi:10.5713/ajas.2007.1278.

[3] D. Yang, A. Lu, D. Ren, J. Wang, Rapid determination of biogenic amines in cooked beef using hyperspectral imaging with sparse representation algorithm, Infrared Phys. Technol. 86 (2017) 23–34. doi:10.1016/j.infrared.2017.08.013.

[4] R.G. Leuschner, M. Heidel, W.P. Hammes, Histamine and tyramine degradation by food fermenting microorganisms, Int. J. Food Microbiol. 39 (1998) 1–10. doi:10.1016/S0168-1605(97)00109-8.

[5] C.A. Lázaro, C.A. Conte-Júnior, A.C. Canto, M.L.G. Monteiro, B. Costa-Lima, A.G. da Cruz, E.T. Mársico, R.M. Franco, Biogenic amines as bacterial quality indicators in different poultry meat species, LWT - Food Sci. Technol. 60 (2015) 15–21. doi:10.1016/j.lwt.2014.09.025.

[6] A.R. Shalaby, Significance of biogenic amines to food safety and human health, Food Res. Int. 29 (1996) 675–690. doi:10.1016/S0963-9969(96)00066-X.

[7] J.L. Mietz, E. Karmas, Chemical quality index of canned tuna as determined by high-pressure liquid chromatography, J. Food Sci. 42 (1977) 155–158. doi:10.1111/j.1365-2621.1977.tb01240.x.

[8] M.T. Veciana-Nogués, A. Mariné-Font, M.C. Vidal-Carou, Biogenic amines as hygienic quality indicators of tuna. relationships with microbial counts, atp-related compounds, volatile amines, and organoleptic changes, J. Agric. Food Chem. 45 (1997) 2036–2041. doi:10.1021/jf960911l.

[9] C.M. Silva, M.B.A. Glória, Bioactive amines in chicken breast and thigh after slaughter and during storage at 4±1 °C and in chicken-based meat products, Food Chem. 78 (2002) 241–248. doi:10.1016/S0308-8146(01)00404-6.

[10] F.B. Erim, Recent analytical approaches to the analysis of biogenic amines in food samples, TrAC - Trends Anal. Chem. 52 (2013) 239–247. doi:10.1016/j.trac.2013.05.018.

[11] J. Płonka, Food analysis - Samples preparation and chromatographic methods in determination of selected biogenic amines, methylxanthines and water-soluble vitamins, Anal. Methods. 4 (2012) 3071–3094. doi:10.1039/c2ay25706h.

[12] E. Dadáková, M. Křížek, T. Pelikánová, Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC), Food Chem. 116 (2009) 365–370. doi:10.1016/J.FOODCHEM.2009.02.018.

[13] J. Płotka-Wasylka, V. Simeonov, J. Namieśnik, An in situ derivatization - dispersive liquid-liquid microextraction combined with gas-chromatography - mass spectrometry for determining biogenic amines in home-made fermented alcoholic drinks, J. Chromatogr. A. 1453 (2016) 10–18. doi:10.1016/j.chroma.2016.05.052.

[14] A. Gałuszka, Z. Migaszewski, J. Namieśnik, The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, TrAC Trends Anal. Chem. 50 (2013) 78–84. doi:10.1016/J.TRAC.2013.04.010.

[15] M. Kamankesh, A. Mohammadi, H. Hosseini, Z. Modarres Tehrani, Rapid determination of polycyclic aromatic hydrocarbons in grilled meat using microwave-assisted extraction and dispersive liquid–liquid microextraction coupled to gas chromatography–mass spectrometry, Meat Sci. 103 (2015) 61–67. doi:10.1016/J.MEATSCI.2015.01.001.

[16] C. Almeida, J.O. Fernandes, S.C. Cunha, A novel dispersive liquid–liquid microextraction (DLLME) gas chromatography-mass spectrometry (GC–MS) method for the determination of eighteen biogenic amines in beer, Food Control. 25 (2012) 380–388. doi:10.1016/J.FOODCONT.2011.10.052.

[17] H. Chen, H. Chen, J. Ying, J. Huang, L. Liao, Dispersive liquid–liquid microextraction followed by high-performance liquid chromatography as an efficient and sensitive technique for simultaneous determination of chloramphenicol and thiamphenicol in honey, Anal. Chim. Acta. 632 (2009) 80–85. doi:10.1016/J.ACA.2008.10.068.

[18] J. Płotka-Wasylka, V. Simeonov, J. Namieśnik, Characterization of home-made and regional fruit wines by evaluation of correlation between selected chemical parameters, Microchem. J. 140 (2018) 66–73. doi:10.1016/J.MICROC.2018.04.010.

[19] J. Płotka-Wasylka, V. Simeonov, J. Namieśnik, Evaluation of the Impact of Storage Conditions on the Biogenic Amines Profile in Opened Wine Bottles, Molecules. 23 (2018) 1130. doi:10.3390/molecules23051130.

[20] J. Demšar, T. Curk, A. Erjavec, T. Hočevar, M. Milutinovič, M. Možina, M. Polajnar, M. Toplak, A. Starič, M. Stajdohar, L. Umek, L. Zagar, J. Zbontar, M. Zitnik, B. Zupan, Orange: Data Mining Toolbox in Python, J. Mach. Learn. Res. 14 (2013) 23492353.

[21] K. Kira, L. Rendell, The Feature Selection Problem: Traditional Methods and a New Algorithm, in: 10th Natl. Conf. Artif. Intell., AAAI Press, San Jose, 1992: pp. 129–134. https://dl.acm.org/citation.cfm?id=1867155 (accessed June 19, 2018).

[22] A. Todorov, An Overview of the RELIEF Algorithm and Advancements, in: M. Windle (Ed.), Stat. Approaches to Gene X Environ. Interact. Complex Phenotypes, MIT Press, 2016.

[23] W. Wojnowski, T. Majchrzak, T. Dymerski, J. Gębicki, J. Namieśnik, Poultry meat freshness evaluation using electronic nose technology and ultra-fast gas chromatography, Monatshefte Für Chemie - Chem. Mon. 148 (2017) 1631–1637. doi:10.1007/s00706-017-1969-x.

[24] A. Gałuszka, Z.M. Migaszewski, P. Konieczka, J. Namieśnik, Analytical Eco-Scale for assessing the greenness of analytical procedures, TrAC Trends Anal. Chem. 37 (2012) 61–72. doi:10.1016/J.TRAC.2012.03.013.

[25] J. Płotka-Wasylka, A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index, Talanta. 181 (2018) 204–209. doi:10.1016/J.TALANTA.2018.01.013.

[26] N. Sayem, E. Daher, R.E. Simard, Putrefactive Amine Changes in Relation to Microbial Counts of Ground Beef During Storage, J. Food Prot. 48 (1985) 54–58.

[27] F. Galgano, F. Favati, M. Bonadio, V. Lorusso, P. Romano, Role of biogenic amines as index of freshness in beef meat packed with different biopolymeric materials, Food Res. Int. 42 (2009) 1147–1152. doi:10.1016/J.FOODRES.2009.05.012.

[28] C.C. Balamatsia, E.K. Paleologos, M.G. Kontominas, I.N. Savvaidis, Correlation between microbial flora, sensory changes and biogenic amines formation in fresh chicken meat stored

aerobically or under modified atmosphere packaging at 4 °C: Possible role of biogenic amines as spoilage indicators, Anton. Leeuw. Int. J. G. 89 (2006) 9–17. doi:10.1007/s10482-005-9003-4.

[29] M.T. Veciana-Nogués, A. Mariné-Font, M.C. Vidal-Carou, Biogenic amines as hygienic quality indicators of tuna. relationships with microbial counts, atp-related compounds, volatile amines, and organoleptic changes, J. Agric. Food Chem. 45 (1997) 2036–2041. doi:10.1021/jf960911l.

[30] G. Vinci, M.L. Antonelli, Biogenic amines: Quality index of freshness in red and white meat, Food Control. 13 (2002) 519–524. doi:10.1016/S0956-7135(02)00031-2.

[31] W. Wojnowski, J. Płotka-Wasylka, K. Kalinowska, T. Majchrzak, T. Dymerski, P. Szweda, J. Namieśnik, Direct determination of cadaverine in the volatile fraction of aerobically stored chicken breast samples, Monatshefte Für Chemie - Chem. Mon. 149 (2018) 1521-1525. doi:10.1007/s00706-018-2218-7.

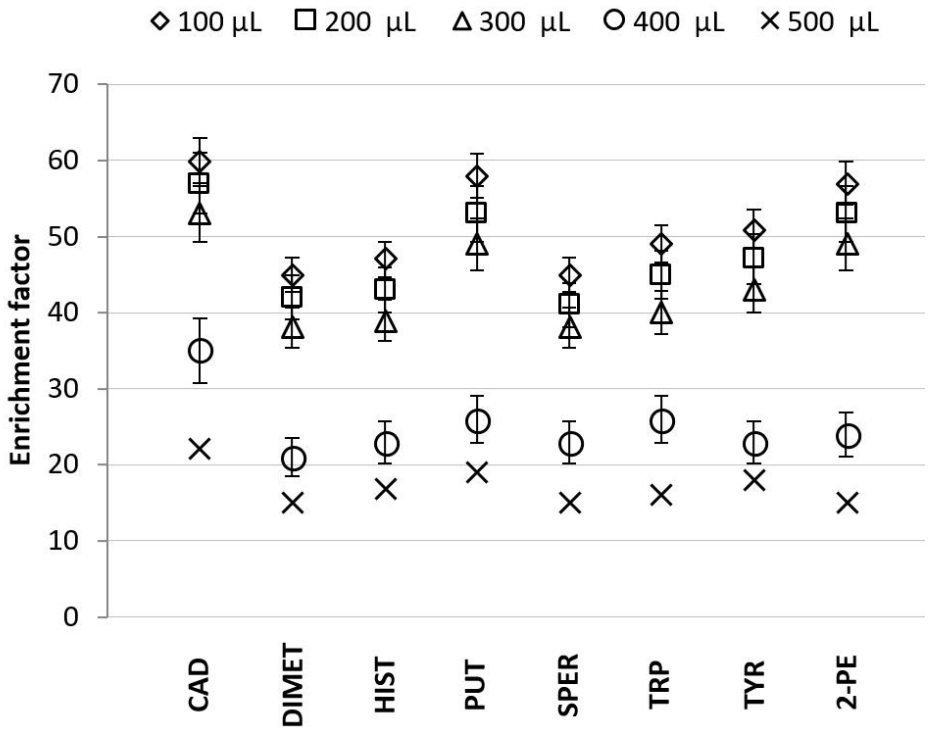
[32] J. Demšar, G. Leban, B. Zupan, FreeViz—An intelligent multivariate visualization approach to explorative analysis of biomedical data, J. Biomed. Inform. 40 (2007) 661–671. doi:10.1016/J.JBI.2007.03.010.

499

500

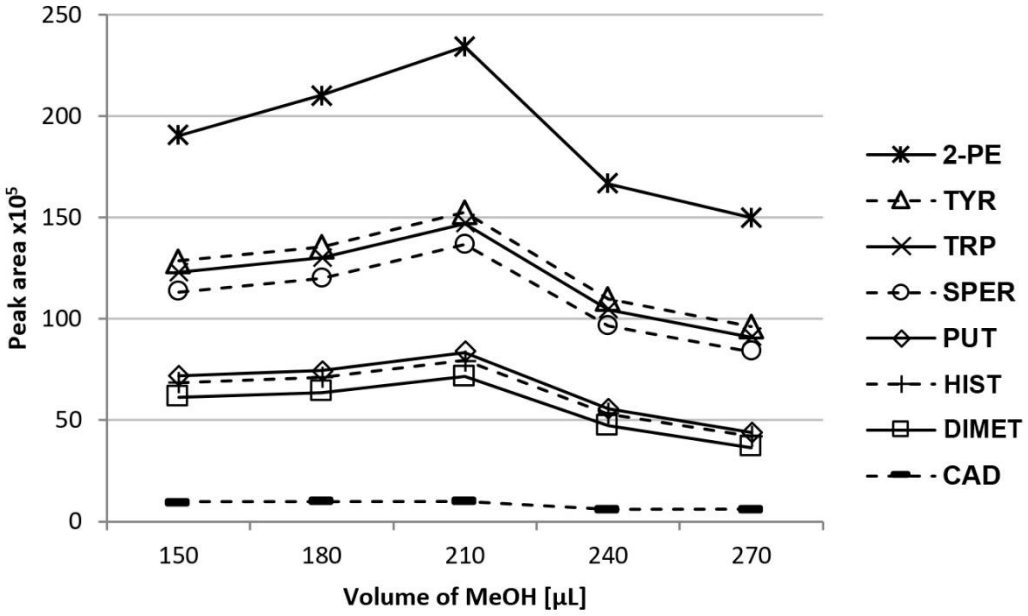
501

502 **Figures:**



503

504 Fig. 1. Enrichment factors obtained using different volumes of extractive solvent, i.e. chloroform (mean
505 value; *n* = 3; RSD < 3.3%).



506

507 Fig. 2. Impact of the volume of methanol on the peak area of BAs by DLLME-GC-MS.

DLLME-GC-MS	
Reagents	**PPs**
Pyridine (55 µL)	1
HCl (55 µL)	3
Chloroform (1 mL)	2
Isobutyl chloroformate (200 µL)	6
MeOH (600 µL)	6
NaOH (5 mL)	1
NaCl	0
	Σ 19
Instruments	**PPs**
Transport	1
GC-MS	2
Occupational hazard	0
Waste	3
	Σ 6
Total PPs: 25	
Score: 75	

UPLC-UV	
Reagents	**PPs**
Acetonitrile (1.5 mL)	4
Dansyl chloride (2 mL)	8
Proline solution (200 µL)	1
Heptane (3 ml)	8
Acetone (3 mL)	4
Na_2CO_3 (0.75 mL)	0
$NaHCO_3$ (0.75 mL)	0
K_2CO_3 (1 mL)	0
$HClO_4$ (<100 mL)	2
	Σ 27
Instruments	**PPs**
Transport	1
UPLC-UV	2
Occupational hazard	1
Waste	5
	Σ 9
Total PPs: 36	
Score: 64	

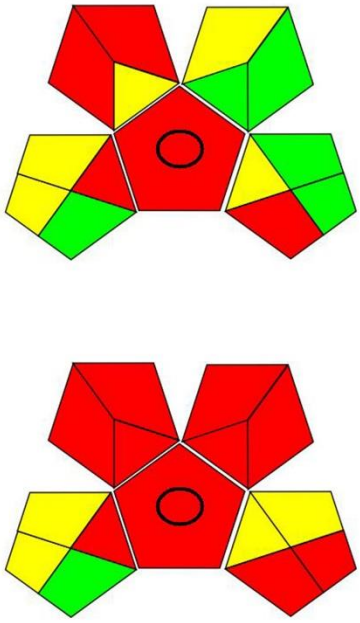


Fig. 3. The penalty points (PPs) for BAs determination in meat samples by in-situ derivatization coupled to DLLME-GC-MS procedure reported in this study and in a different reported procedure [12].

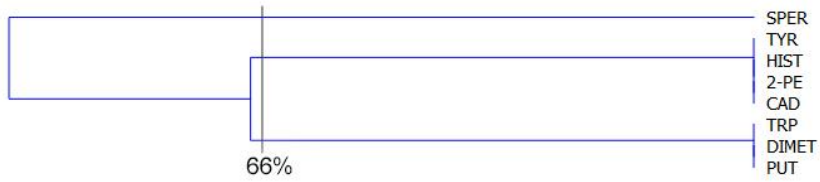


Fig. 4. Hierarchical cluster analysis of the variables used in the data analysis

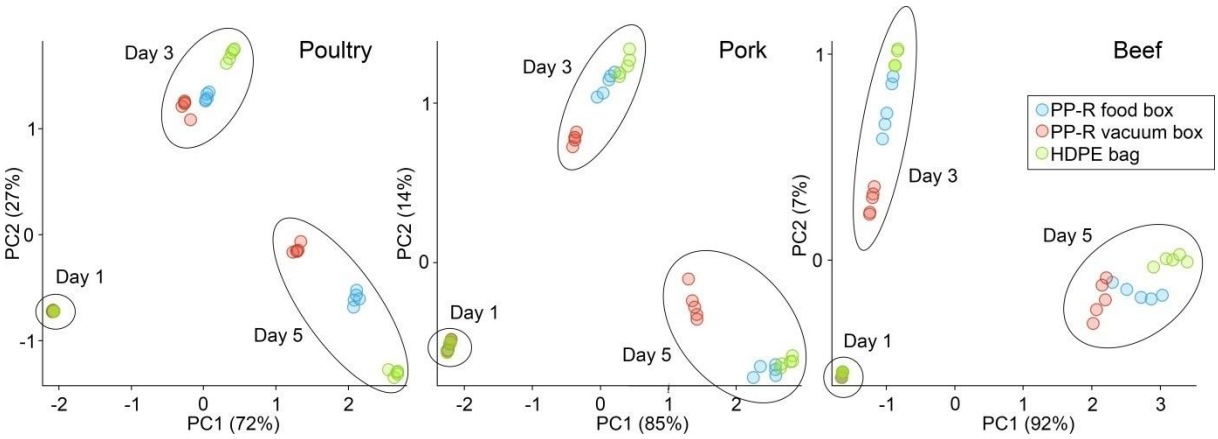
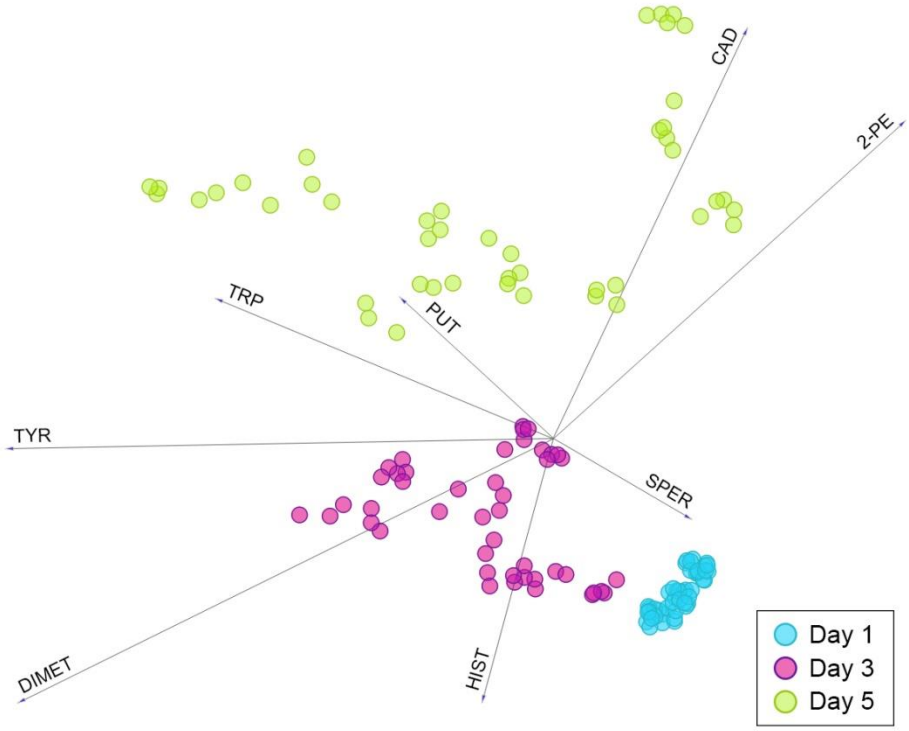


Fig. 5. Principal component analysis of the concentration values of BAs in meat samples according to the duration of refrigerated storage and packaging material. In all 3 cases, the first two principal components cover 99 % of the total variance.



518

519 Fig. 6. Linear projection of the variables in the classification of poultry, pork and bovine meat based on
520 the duration of refrigerated storage.

521