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-Green, simple analytical method for biogenic amines determination in fruit juice samples using salting-out assisted liquid-liquid microextraction and gas chromatography-mass spectrometry

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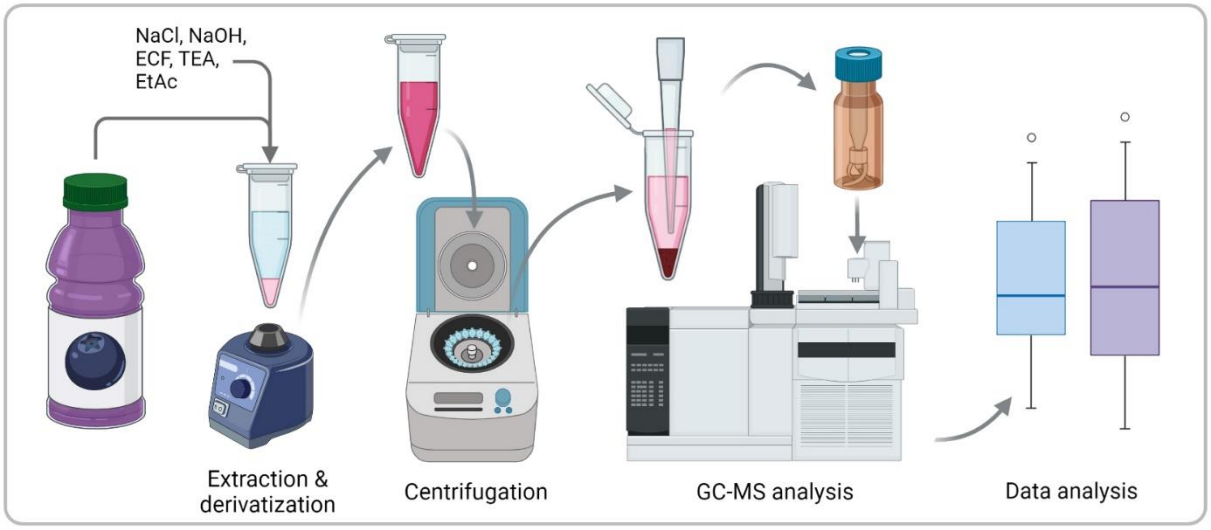
Highlights:

- A novel SALLME-GC-MS method was developed for BAs determination in fruit juices.
- One-step extraction and derivatization protocol for BAs were established.
- RSM was employed for the optimization of SALLME parameters.
- Low LODs and LOQs, and good recoveries and reproducibility were obtained.
- The green character of the method was assessed.

Abstract

Salting-out assisted liquid-liquid microextraction (SALLME) was integrated with the derivatization procedure to establish a one-step sample pre-treatment approach for rapid analysis of 14 biogenic amines (BAs) in fruit juices. The methodology consists of salting-out of analytes, derivatization with ethyl chloroformate (ECF), extraction with ethyl acetate (EtAc), and the analysis of the derivatized BAs using gas chromatography-mass spectrometry (GC-MS). Optimization of the SALLME parameters, including the amount of sample, NaOH, and ECF was carried out through a Box-Behnken response surface design. The developed method exhibits satisfactory limits of detection (from 1.5 to 8.1 µg/L) and quantification (from 5.0 to 26.7 µg/L), and average recoveries between 84% and 108%. The developed procedure was used for BAs determination in juices of different berries with the highest determined concentrations found for cadaverine, putrescine, tryptamine, and tyramine. Both GAPI and AGREE tools were used to assess the green character of the SALLME-GC-MS procedure.

Graphical abstract



Keywords: biogenic amines, salting-out assisted liquid-liquid extraction, gas chromatography-mass spectrometry, experimental design, fruit juices, greenness evaluation

1. Introduction

Biogenic amines (BAs) are nitrogenous organic bases formed in food as a result of the activity of microorganisms capable of decarboxylating amino acids (Wójcik et al., 2021). BAs are commonly found in food products, especially those subjected to fermentation processes (beer, wine), long maturation (cheese), rich in protein (meat, fish), but also fresh fruits and vegetables (Wójcik et al., 2021). Consumption of BAs in low concentrations is not dangerous to the consumers' health. However, consumed in excess, they can cause several toxic effects such as nausea, vomiting, diarrhoea, headache, respiratory failure, and palpitations (Doeun, Davaatseren, & Chung, 2017). In the case of non-fermented beverages, such as e.g. juices, a high BAs content may indicate undesirable activity of microorganisms (Vinci & Maddaloni, 2020). Therefore, BAs concentration can be a useful indicator of food quality and safety (Ruiz-Capillas & Herrero, 2019).

Biogenic amines determinations are commonly performed using ion chromatography (Jastrzębska, Piasta, & Szłyk, 2015), liquid chromatography (Eliassen, Reistad, Risoen, & Ronning, 2002; Saaid, Saad, Hashim, Mohamed Ali, & Saleh, 2009), and gas chromatography (Cunha, Faria, & Fernandes, 2011; Fernandes & Ferreira, 2000) coupled with various detectors. When developing new analytical methods for the determination of BAs in food, several problems should be bore in mind, namely the low concentration of analytes, the presence of many interfering substances, the complex matrix composition, and the polar nature of BAs. To obtain satisfactory results, the procedure of extraction and derivatization of analytes should be properly selected (Płotka-Wasylka, Morrison, Biziuk, & Namieśnik, 2015).

The most commonly used reagent to derivatize BAs from fruit juice samples is dansyl chloride, since it forms very stable derivatives and can react with both primary and secondary amines (Basheer et al., 2011). The products of such derivatization are then analysed using high-performance liquid chromatography coupled with spectrophotometric or fluorometric detection. Other frequently used BAs derivatizing agents are o-phtaldialdehyde (Vieira, Theodoro, & Glória, 2007), 1-naphthylisothiocyanate (Jain, Gupta, & Verma, 2015), heptafluorobutyric acid (Fernandes & Ferreira, 2000), and isobutyl chloroformate (Cunha et al., 2011). Alkyl chloroformates are extremely useful in determining BAs using GC-MS because the derivatization step avoids the low sensitivity and the tailing of the peaks due to the high polarity of the amines (Zaikin & Halket, 2003). Moreover, the derivatization process with alkyl chloroformates is inexpensive and less time-consuming compared to derivatization with dansyl chloride. Alkyl chloroformates are reactive in an aqueous medium facilitating their use in the analysis of beverages samples.

The next critical step in the analytical process is the extraction of the analytes. To isolate BAs prior to chromatographic analysis, conventional liquid-liquid extraction (LLE) is most often used (Kelly, Blaise, & Larroque, 2010; Preti, Antonelli, Bernacchia, & Vinci, 2015). However, it is characterized by high consumption of hazardous reagents and solvents. To reduce the consumption of chemicals, as well as the produced wastes, other techniques such as dispersive liquid-liquid microextraction (DLLME) (Cunha et al., 2011) and micro-solid phase extraction (μSPE) (Tameem, Saad, Makahleh, Salhin, & Saleh, 2010) have been proposed in the literature.

However, multi-step extraction and subsequent derivatization of analytes can result in loss of analytes and increase the measurement uncertainty. The solution could be a simple and fast salting-out assisted liquid-liquid extraction (SALLE) with simultaneous derivatization. The salting-out effect consists of adding an electrolyte to an aqueous solution to change the ionic strength of the mixture, which favours the extraction of the analytes into the organic phase (Tsochatzis, Lopes, Gika, Dalsgaard, & Theodoridis, 2021). SALLE has been used for the extraction of BAs from samples of cheese (Ramos, Brandão, & Rodrigues, 2020), meat, fish (Francisco et al., 2020), and wines (Ramos, Valente, & Rodrigues, 2014).

This study was aimed at developing a simple, fast, and green SALLME procedure for the extraction of BAs from fruit juices, followed by the use of GC-MS for identification and quantification. The optimization of the method was carried out using the Design of Experiment (DoE) instead of the commonly used one-time-factor procedure. The three major parameters influencing SALLME were analysed using Box-Behnken Design to determine the optimal extraction conditions. Then, accuracy and precision of the developed method were estimated and validated. The method's greenness was assessed using two analytical tools: GAPI (Płotka-Wasylka, 2018) and AGREE (Pena-Pereira, Wojnowski, & Tobiszewski, 2020). Finally, various fruit juices from local grocery stores were

analysed to demonstrate the applicability of the developed methodology for the determination of BAs in food products. To the best of our knowledge, this is the first work focused on the application of *in situ* derivatization coupled with SALLME for the determination of biogenic amines in fruit samples by GC-MS. The procedure is characterized by good validation and separation parameters, and also conforms with many criteria of Green Analytical Chemistry.

2. Materials and methods

2.1. Reagents, chemicals and standards

All the BAs, such as methylamine hydrochloride (MET, 98.0%), dimethylamine hydrochloride (DIMET, 99.0%), ethylamine hydrochloride (ET, 98.0%), diethylamine hydrochloride (DIET, 99.0%), propylamine (PROP, 98.0%), butylamine (BUT, 99.5%), isopentylamine (IPA, 98.0%), hexylamine (HEX, 99.0%), 2-phenylethylamine hydrochloride (2PEA, 98.0%), putrescine (PUT, 97.5%), cadaverine (CAD, 96.5%), histamine (HIS, 96.5%), tyramine (TYR, 96.5%) and tryptamine (TRYP, 97.5%) were purchased from Merck (Merck Life Science Sp.z.o.o, Poznań, Poland). Aniline (IS, 99.5%), ethyl chloroformate (ECF, 99.5%) and triethylamine (TEA, 99.5%) which were used as internal standard, derivatizating agent and catalyst respectively were also from Merck Life Science Sp.z.o.o (Merck, Poznań, Poland). Ultrapure water for aqueous solutions and glassware washing was prepared using HLP5 Hydrolab (Wiślina, Poland). For the salting-out assisted liquid-liquid microextraction procedure, sodium chloride (NaCl, ACS grade, POCH, Gliwice, Poland), sodium hydroxide (NaOH, ACS grade, POCH, Gliwice, Poland) together with ethyl acetate (EtAc, 99.9%, VWR International, Gdańsk, Poland) were used.

2.2. Samples and preparation procedure

The nonfiltered berry juice samples used in this work were commercially available in local supermarkets and were of different types: bilberry juice (BI), blackcurrant juice (BL), blueberry juice (BU), chokeberry juice (CH), elderberry juice (EL), honeyberry juice (HO) and raspberry juice (RA). Juice samples of the same type were purchased from several producers. All samples were stored at refrigerator temperature and were protected from light.

All samples were derivatized according to SALLME protocol. In a 2 mL Eppendorf tube, 32.5 mg of sodium chloride and 65 µL of fruit juice (enriched with 1.3 µL of IS at a concentration of 50 µg/mL) were mixed. Afterwards, ECF derivatization combined with simultaneous liquid-liquid extraction (LLE) was carried out at room temperature. More in detail, 11 µl of NaOH solution (1.0 mol/L) were added to the sample to obtain the pH appropriate for carbamate formation, followed by the addition of 1.2 µL of the derivatizing reagent (ECF) and 1.2 µL of TEA. The solution was mixed with 260 µL of EtAc and then vortexed for 1 min at 2500 rpm. Then, the mixtures in the Eppendorf

tubes were centrifuged for 2 min at 3500 rpm. Finally, an aliquot of the upper organic phase was collected and analysed by GC-MS. Each sample was prepared in five replicates. A schematic representation of the derivatization and extraction process is presented in Fig. 1.

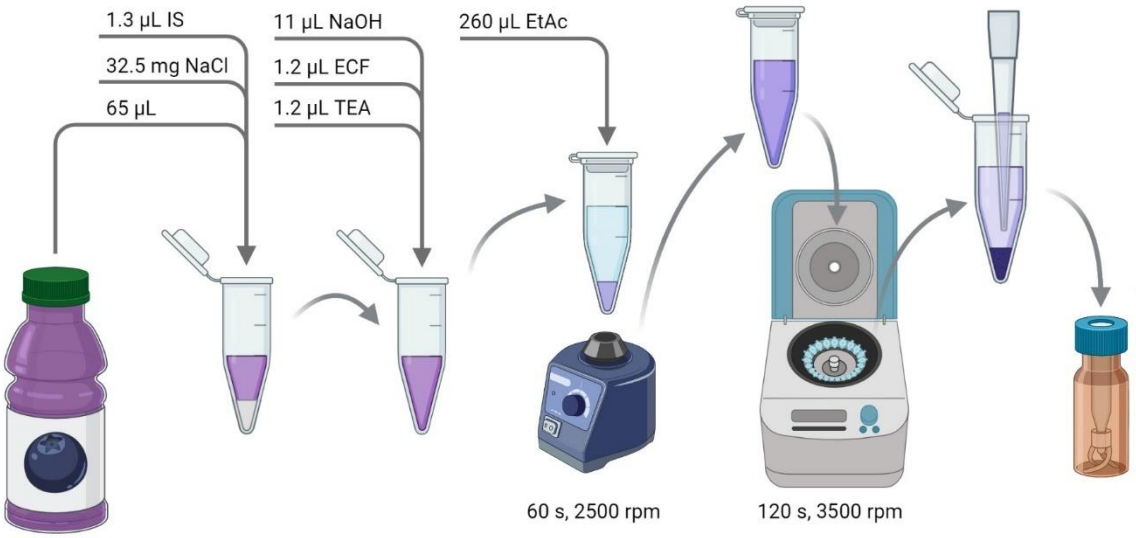


Fig. 1 Scheme of SALLME protocol for BAs analysis in juice samples

2.3. Instrumentation

All analyses were carried out on a GC-MS instrument (Agilent Technologies, Santa Clara, CA, USA) consisting of an Agilent 7890A gas chromatograph coupled to an Agilent 5975C single quadrupole mass spectrometer detector. A fused silica capillary column (0.3 m × 0.25 mm, Phenomenex, Torrance, CA, USA) was used as a guard column connected to a ZB-5MS capillary column (0.3 m × 0.25 mm × 0.25 µm, Zebron, Phenomenex, Torrance, CA). Helium (99.999% pure, Air Liquide, Kraków, Poland) was used as a carrier gas with a flow rate of 1.0 mL/min. 2 µL of the extracts were transferred into an injector which was operated in splitless mode at 240 °C. For the chromatographic separation, the GC oven temperature program was as follows: initial temperature 55 °C, held for 4 min, then increased to 280 °C at 50 °C/min and held for 7.5 min. All targeted compounds were separated within 16 min. The total time needed for analysis per sample was 20 min: 1.0 min for reagents preparing, 1.0 min for derivatization and extraction step, 2.0 min for centrifugation and 16.0 min for the chromatographic run. The MS was performed in EI mode (70 eV). The transfer line, ion source and detector temperatures were 300 °C, 230 °C and 150 °C, respectively. Synchronous scan/selected ion monitoring (SIM) mode was used for the collection of both types of data in each run (solvent delay: 4.5 min). The scan m/z range was set to 30–500 amu. In the SIM mode, one quantification and two qualifier ions were monitored for quantification purposes. Data were acquired using MSD ChemStation, Ver. E.02.00.493 software from Agilent

Technologies. For the preparation step Vortex (MX-S, (Chemland, Stargard, Poland) and mini-centrifuge (FVL-2400N Combi-Spin, Biosan, Józefów, Poland) were utilized.

2.4. Box-Behnken design

The optimization of biogenic amines extraction was performed using the response surface methodology (RSM) (Minitab 17, LLC, State College, Pennsylvania, USA). The design of experiment (DoE), namely the Box-Behnken design, was used to evaluate the optimal level and interaction effects of the three independent factors affecting the content of the BAs. The three experimental factors and factor levels were selected by preliminary studies based on the results of single-factor tests (data not shown). Factors in question were sample volume [Sample], the volume of NaOH solution (1 mol/L) [NaOH] and the volume of derivatizing reagent [ECF]. A total of 18 experiments (3-level design including runs in the full three-level factorial and 6 centre points to estimate the experimental error) were carried out. During randomized runs order the juice samples enriched with BAs mix at 10 µg/mL were analysed. The response value was depicted by the total sum of the standardized peak areas for BAs. To interpret the effects of these independent variables on BAs extraction efficiency, three-dimensional response surface plots were constructed. RSM was implemented to optimize the extraction process. The regression coefficients of linear, quadratic, and interaction involved in the model and their effects were tested statistically by one-way analysis of variance (ANOVA) at probability levels ($p \leq 0.05$). Graphical and numerical analyses were used to optimize the processing conditions based on the model desirability features.

2.5. Quality assurance (QA)

The optimized method was evaluated using the following validation parameters: linearity, precision, sensitivity and accuracy according to quality assurance protocol. Linearity was examined by application of 10 different concentrations. Limits of detection (LODs) and limits of quantification (LOQs) were calculated to estimate the sensitivity of the methodology. Both LODs and LOQs were calculated from spiked samples (n=5) and the minimum detectable analyte amount with a signal-to-noise ratio of 3 and 10, respectively, was established. The intra-day (RSD_r) and inter-day (RSD_R) precision were determined by the application of five replicates of juice samples spiked at two levels (0.25 and 2.5 mg/L). In addition to validation parameters, recovery rates were estimated using the ratio of the peak areas of the spiked samples of known concentration of biogenic amines to those of spiked ethyl acetate solution (n=5).

2.6. Data analysis

Chromatographic data were processed using MZmine 2 (Pluskal, Castillo, Villar-Briones, & Orešič, 2010). The concentration values of the BAs determined in fruit juice samples were used as input data for multivariate statistical data analysis using a dedicated Python toolkit Orange v.3.20. Initial data processing involved standardization. Standardized values were taken as input to the cluster analysis and principal component analysis. Based on its results the heat map, loadings plot and linear projection for the three principal components were obtained.

3. Results and discussion

3.1. Analytical method development

3.1.1. Optimization of SALLME and derivatization protocol

The main parameters affecting the efficiency of SALLME are sample volume, type and volume of extractant, duration of extraction, process temperature, and type and amount of added salt (Jain et al., 2015; Ramos et al., 2014). In the proposed procedure, the extraction and derivatization processes were carried out simultaneously which significantly shortens the sample preparation step, but also introduces additional factors that can affect the efficiency of the extraction. Proper selection of the type and volume of the derivatizing agent as well as ensuring that the solution is basic, not only facilitates the extraction but also enables the formation of the products of the derivatization (Husek & Simek, 2006; Munir & Badri, 2020).

In SALLME, the salting-out effect is used to facilitate the separation of water-miscible organic solvents by adding electrolytes to the solution, which changes the ionic strength and increases extraction efficiency (Francisco et al., 2020; Tsochatzis, Lopes, Gika, Dalsgaard, & Theodoridis, 2021). The main factor responsible for the efficiency of phase separation in SALLME procedures is the type of salt anion (Ramos et al., 2020). Another important aspect that needs to be taken under consideration is the fact that the amount of added salt must be sufficient for clear separation of the two phases, but its amount should be as small as possible to avoid adsorption of the analytes on the salt crystals surface. (Tsochatzis et al., 2021). For this reason, the NaCl amount corresponding to 10% w/v of the reaction mixture was chosen. Another crucial factor is the type and volume of the extractant. Ethyl acetate was chosen as the extraction solvent because of its green nature and the fact that it is immiscible both with water and juice samples (Manca et al., 2017; Sánchez, Santos, Sappó, Pavón, & Cordero, 2014). The aim was to develop an extraction procedure performed on a micro-scale, therefore it was decided that the total volume during extraction should not exceed 1 mL. Assuming a suitable solvent to sample ratio of 4:1 (Fabio et al., 2020), 200 to 600 µL of EtAc was used.

In the case of BAs determination using GC-MS, derivatization is carried out in order to change the nature of the analytes, improve the shapes of the chromatographic peaks, increase the sensitivity

and selectivity of the technique, and improve the quality of the mass spectra. The derivatization process was performed using different alkyl chloroformates, namely methyl chloroformate (MCF), ethyl chloroformate (ECF), propyl chloroformate (PCF), and isobutyl chloroformate (IBCF). The derivatization agent was chosen based on the results obtained during GC-MS analysis of four BAs, selected according to their chemical structure and derivatized with the above-mentioned chloroformates (Fig. 2).

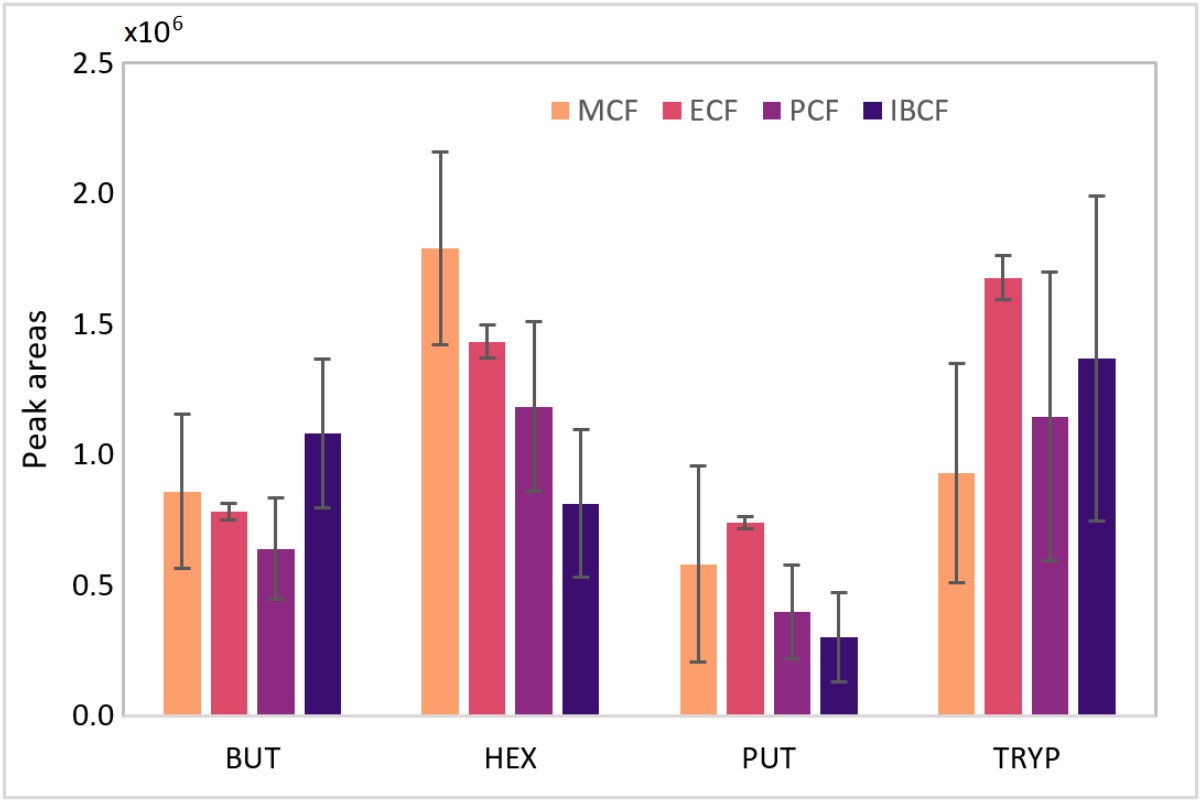


Fig. 2 Influence of the derivatization reagent on the resulting peak areas of selected biogenic amines; MCF – methyl chloroformate, ECF – ethyl chloroformate, PCF – propyl chloroformate, IBCF – isobutyl chloroformate

The results for the BAs derivatized with ECF were characterized with the lowest SD, and for PUT and TRYP, the peak areas were the largest, therefore it was selected as a derivatization reagent for further stages of the research. The pH of the sample is a parameter that should be controlled before the derivatization process as it affects the time of the reaction. Moreover, in the case of reaction with chloroformates, amines must be in the deprotonated form before the derivatization step (Husek & Simek, 2006; Qiu et al., 2007; Zaikin & Halket, 2003). To this end, optimized amounts of NaOH solution (1mol/L) were added to the samples to improve the efficiency and shorten the time of derivatization with ECF. The optimum pH of the acylation reaction and the formation of carbamates is pH>10 (Hušek, 1998; Husek & Simek, 2006). Fruit juices are acidic, therefore during the sample preparation process, an optimized volume of NaOH solution

(1mol/L) was added to increase the pH to basic. After adjusting the pH, an optimized volume of ECF was added to the sample. Both parameters, volume of NaOH and ECF were selected for optimization using DoE (Section 3.1.2). TEA was added to the samples to remove the by-product of derivatization, i.e. hydrogen chloride, from the reaction mixture (Husek & Simek, 2006).

3.1.2. Response surface methodology for SALLME and derivatization protocols

The RSM was used to evaluate the maximum efficiency of BAs extraction from food samples. The extraction yield is expressed as the standardized sum of the peak areas. The influence of three independent variables was investigated, namely the sample volume (50-150 µL), the NaOH solution volume (volume corresponding to 5.0-35.0% of the sample volume), and the volume of the derivatizing agent, i.e. ECF (volume corresponding to 0.5-2.5% of the sample volume). The codes and levels of the standardized variables along with the experiment design are listed in Table S1.

A polynomial model for estimating the BAs content in terms of sample volume, NaOH content, and ECF content is shown in Equation 1:

$$BAs = -1.266 + 0.00806\,[Sample] + 0.07552\,[NaOH] + 1.332\,[ECF]$$
$$-0.000039\,[Sample]^2 - 0.001473\,[NaOH]^2 - 0.2460\,[ECF]^2$$
$$+0.000006\,[Sample]\cdot[NaOH] - 0.001562\,[Sample]\cdot[ECF]$$
$$-0.01172\,[NaOH]\cdot[ECF] \qquad (\textit{Equation 1})$$

The results of ANOVA and model coefficients are listed in Table S2. The F-value (45.53) and the associated p-value ($p < 0.001$) indicated that the regression model was significant. The F-value (0.69) for lack of fit was negligible (LoF>0.05) and therefore the validity of the model was confirmed. High values of R^2 (0.981), pred-R^2 (0.959), and adj-R^2 (0.891) indicated the high predictive ability of the model. The low correlation variance (CV) value indicated that the experiments were characterized by a high degree of reliability and precision. Based on the analysis of the p-values of each component of the model, it was possible to conclude that two linear coefficients ([Sample] and [ECF]), three square coefficients ([Sample]^2, [NaOH]^2, [ECF]^2), and two two-way interaction coefficients ([Sample]·[ECF] and [NaOH]·[ECF]) were significant and indicative of a pattern of interactions between the studied variables.

The application of the Box-Behnken design resulted in three response surface plots for BAs extraction, which are graphical representations of the regression equation (Fig. 3). With the use of these plots, it is possible to visualize the relationship between the responses and the experimental parameter levels of variables, and the type of interaction between them.

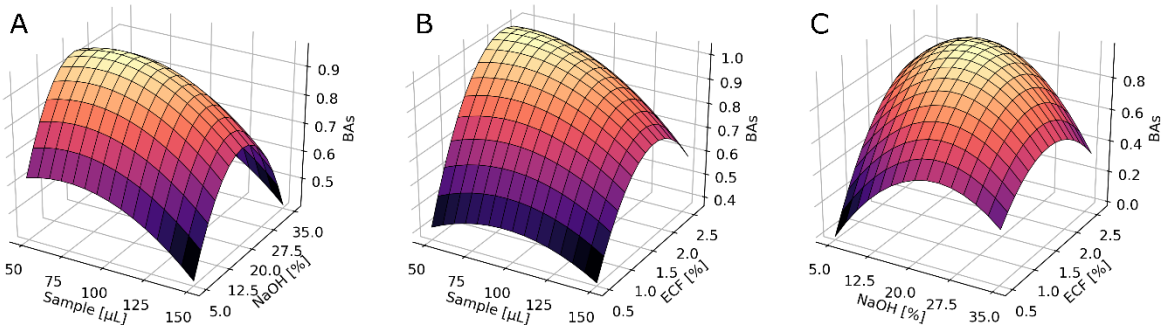


Fig. 3 Response surface plots of Box-Behnken DoE. The x and y-axis represent the variables, namely sample volume, NaOH content and ECF content while the z is the standardized sum of BAs peak areas

In Fig. 3 it can be seen that all three optimized parameters have a large impact on the efficiency of BAs extraction. Extraction efficiency was inversely proportional to the sample volume. The sum of the BAs peak areas increased with increasing NaOH content up to approx. 20%, and then extraction efficiency decreased. There is an inconsistency in the literature about the most appropriate concentration of derivatizing reagents for BAs, as it depends on the type of sample and the solvent (Hušek, 1998; Ramos et al., 2020). Theoretically, the higher the derivatization reagent amount, the more effective the derivatization process. However, excessive volume of alkyl chloroformate can result in the formation of by-products that interfere with BAs derivatives determination and can also shorten the life of the chromatographic system (Munir & Badri, 2020; Zaikin & Halket, 2003). This is why it is important to optimize the volume of the ECF so that the derivatization can be performed efficiently whilst keeping the derivatizing agent volume as low as possible. This is consistent with the obtained results (Fig. 3), where the yield was directly proportional to the ECF content until reaching the value of 2%, beyond which point the efficiency of the process decreased. Based on the obtained results, it was found that the optimal values of the three continuous variables of the BAs extraction and derivatization procedure from juice samples are: 65 µL of the sample, 17% NaOH, and 2.0% ECF. The four experiments were conducted using optimal values and the obtained normalized sum of peak areas for BAs was 1,041 ± 0,014, which was comparable to the predicted value of 1,037 calculated by the model.

3.1.3. Optimization of GC-MS conditions

Gas chromatography method parameters, namely temperature programme, injector temperature, and carrier gas flow, among others, were selected to obtain satisfactory separation and signals for all analysed BAs. Additionally, the goal was to obtain a high-throughput method. The chromatographic run took 16 min. The mass spectrometric conditions were also optimized to ensure the best parameters for BAs analysis. Peak identification was performed by comparing

the retention times and MS spectral information with the information obtained from the analysis of standard solutions. It is worth noting that not all BAs bind to a single molecule of ECF. Based on the obtained results, it can be observed that monoamines bind to only one ECF molecule, while polyamines bind to one or two ECF molecules. This information, along with retention times and characteristic fragments obtained, can be found in Table S3. The molecular ion peaks were observed in the mass spectra of all analytes. The m/z 102 fragment appears in the mass spectra of most of the analysed amines. This fragment can be related to the common presence of the $CH_3CH_2OC(O)NHCH_2$ group in the molecular structures of these compounds, i.e. (N-methyl)-ethyl carbamate group (Reddy, Chary, Pavankumar, & Prabhakar, 2016).

3.2. Method validation

The matrix effect (ME) is one of the main challenges when developing new analytical methods. For this reason, the ME of the optimized method was evaluated using the procedure described by Matuszewski et al. (Matuszewski, Constanzer, & Chavez-Eng, 2003). The ME was tested at a concentration level of 0.25 mg/L, and calculated by comparing the mean peak area of the analyte standards in the EtAc solution (A, *n=5*) with the mean peak area of an analyte spiked postextraction (B, *n=5*). The following Equation was used:

$$ME\ [\%] = \frac{B}{A} \cdot 100\% \qquad \textit{(Equation 2)}$$

The MEs, shown in Table 1, were ranged from 82% and 101%. In general, ME has no impact on the qualitative and quantitative results of this method and can be omitted. Additionally, it was proven that it is justified to use an internal standard (IS) for calibration.

The method's linearity and sensitivity were assessed for fourteen BAs by calibration with standard solutions in the presence of IS. Ten EtAc solutions containing all tested BAs in two separate concentration ranges: from 0.05 to 1 mg/L and from 1 to 10 mg/L, respectively, were subjected to the developed procedure. Least squares linear regression was used to calculate the equations of the calibration curves and the determination coefficients (R^2). Detailed information for each analyte is provided in Table 1. The linearity was excellent for all analytes with determination coefficients from 0.9948 to 0.9989 (for the first range) and from 0.9956 to 0.9993 (for the second range). The LODs ranged from 1.5 to 8.1 µg/L and the LOQs ranged from 26.7 to 49.5 µg/L. LOD and LOQ were the lowest for CAD and the highest for PUT.

329 *Table 1 Analytical figures of merit for the developed SALLME-GC-MS methodology.*

Analyte	Concentration range		Concentration level				Inter-day (%RSD)			LOD (µg/L)	LOQ (µg/L)	ME (%*RSD*)
			0.25 mg/L		2.5 mg/L							
	0.05 to 1 mg/L	1 to 10 mg/L	Intra-day (%RSD)	Recovery (%)	Intra-day (%RSD)	Recovery (%)	Day 1	Day 2	Day 3			
	Linearity (R^2)											
MET	0.9956	0.9965	3.3	91	3.5	95	4.4	4.4	4.6	2.3	7.6	93 (4.1)
DIMET	0.9978	0.9985	6.2	93	6.8	97	6.5	6.4	6.7	5.4	17.8	95 (5.0
ET	0.9968	0.9977	4.1	96	3.3	99	4.8	4.9	4.7	2.3	7.6	96 (9.8)
DIET	0.9948	0.9956	10.4	99	10.9	100	11.3	11.5	11.6	1.9	6.3	101 (9.3)
PROP	0.9987	0.9991	2.3	84	2.6	91	2.5	2.5	2.7	4.2	13.9	83 (6.9)
BUT	0.9979	0.9989	4.7	101	4.9	99	4.3	4.5	4.6	2.8	9.2	95 (7.1)
IPA	0.9983	0.9987	3.1	92	3.6	96	3.4	3.1	3.3	5.3	17.5	96 (8.3)
HEX	0.9987	0.9992	6.0	97	5.9	102	6.3	6.6	6.4	2.7	8.9	93 (5.4)
2PEA	0.9986	0.9990	4.3	99	5.0	98	4.2	4.4	4.5	6.4	21.1	94 (7.2)
PUT	0.9980	0.9991	4.1	101	4.9	102	4.4	4.7	4.6	8.1	26.7	98 (3.9)
CAD	0.9985	0.9993	2.9	85	3.6	89	3.1	3.0	3.3	1.5	5.0	82 (4.1)
HIS	0.9989	0.9993	3.4	95	4.1	97	3.7	3.3	3.7	2.9	9.6	91 (3.7)
TYR	0.9989	0.9988	4.7	96	4.9	98	5.0	4.7	4.9	2.1	6.9	94 (9.1)
TRYP	0.9952	0.9975	10.1	106	11.3	108	10.6	9.9	10.8	3.1	10.2	99 (11.1)

MET – methylamine; DIMET – dimethylamine; ET- ethylamine; DIET – diethylamine; PROP – propylamine; BUT – butylamine; IPA- isopentylamine; HEX – hexylamine; 2PEA – 2-phenylethylamine; PUT – putrescine; CAD – cadaverine; HIS – histamine; TYR – tyramine; TRYP – tryptamine; ME – matrix effect

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The intra-day precision (RSD_r) was estimated based on the results of analysis of five replicates of juice samples fortified at two concentration levels (0.25 and 2.5 mg/L) on the same day. The inter-day precision (RSD_R) was determined by analysis of samples from three different days over three weeks. RSD_r ranged from 2.3 to 10.4% (for 0.25 mg/L) and from 2.6 to 11.3% (for 2.5 mg/L), while RSD_R ranged from 2.5 to 11.6% (Table 1). The stability of the analytes in the juice matrix led to satisfactory precision.

The accuracy of the method was determined by a recovery test, i.e. a comparison of the unenriched sample with the samples enriched with analytes at two concentration levels (0.25 and 2.5 mg/L) with five replicates. The recovery rates are listed in Table 1. The average recovery values ranged from 84 to 106% (for 0.25 mg/L) and from 89 to 102% (for 2.5 mg/L). These results indicate that the developed procedure of BAs determination in fruit juices samples was characterized by high accuracy.

3.3. Greenness evaluation

The developed analytical procedure for the determination of BAs in fruit juice samples was subsequently assessed in terms of 'greenness' using two different metrics, namely the Green Analytical Procedure Index (GAPI) (Płotka-Wasylka, 2018) and the Analytical Greenness Calculator (AGREE) (Pena-Pereira et al., 2020). To evaluate its environmental impact, the developed approach was juxtaposed with five other methods for BAs determination chosen from the literature. Two different analytical methodologies based on GC-MS were selected for the comparison: method denoted M2 in which the analysis proceeded with multi-stage LLE and isobutyl chloroformate derivatization (Cunha et al., 2011) and M3 in which sample preparation consists of ion-pair extraction and heptafluorobutyric anhydride derivatization (Fernandes & Ferreira, 2000). Three liquid chromatography-based methodologies were also included in the greenness evaluation, since LC is seen as the gold standard for BAs determination. In selected methods involved different sample preparation techniques: micro-solid phase extraction (μ-SPE) and dansyl chloride derivatization in M4 (Basheer et al., 2011), conventional LLE also with dansyl chloride derivatization in M5 (Preti, Bernacchia, & Vinci, 2016), and SALLE combined with 1-naphthylisothiocyanate derivatization performed prior to the determination in M6 (Jain et al., 2015).

Based on the obtained results for the greenness assessment (Fig. S1), SALLME-GC-MS method developed in this work is the greenest. Compared to other methods, its main advantage is a very short time of the derivatization and extraction step (only 4 minutes), while in other methodologies it ranges from 25 min (Cunha et al., 2011) to 90 min (Preti et al., 2016). To reduce the negative impact on the environment, the throughput of the methodology was also increased by reducing the analysis time (16 min) and increasing the number of analysed BAs during a single

analysis (14 amines). In addition, the entire extraction procedure was miniaturized, so that only 65 µL of the sample is needed for the analysis, and the amount of waste was reduced to approx. 370 µL per analysis. Therefore, the adoption of the proposed method for the analysis of BAs in analytical laboratories would result in reducing health hazards and environmental impact.

3.4. Real samples analysis

The developed analytical method was used to determine BAs in selected nonfiltered berry juices characterized by a high content of bioactive substances (Table S4). PUT and CAD were detected in each sample which was to be expected since they are typically found in plant-based products (Ordonez & Callejon, 2020). PUT was reported to be the predominant amine in most fruit juice samples (Eliassen et al., 2002). DIMET and DIET were detected only in a few samples. The ranges of each biogenic amine content in berry juices are shown in Fig. 4A. The most abundant amines in fruit juices were TYR with an average concentration of 197±70 µg/L, TRYP (129±88 µg/L), PUT (91±49 µg/L), and CAD (46±27 µg/L). The mean concentrations of the remaining BAs ranged from 8.0 µg/L for DIET to approx. 25 µg/L for 2PEA. Jastrzębska et al. also noted that the most abundant amines in the samples of non-filtered juices were TYR, PUT, and CAD, and their concentration depended on the type of juice (Jastrzębska et al., 2015). Additionally, Saaid et. al. observed that in tropical fruit juices the most abundant amines were TRYP and HIS, while in blackcurrant juice the most abundant amines were PUT, HIS, and spermidine (Saaid et al., 2009). Low levels of PUT in juices may also suggest that overripe fruit were not used in their production (Jastrzębska et al., 2015). The level of TYR can fluctuate during storage even at refrigerator temperatures and a high content of this amine may indicate that a long time has elapsed between the production of the juice and its purchase (Saaid et al., 2009).

The total BAs content depending on the type of juice is shown in Fig. 4B. Based on these results, it can be observed that the samples of honeyberry, blueberry, elderberry, and blackcurrant juices were characterized by a relatively small variation in BAs concentrations. On the other hand, the BAs concentration in raspberry, chokeberry, and bilberry juices had high variability. This may be caused by the fact that the amount of BAs, apart from the storage conditions of food products, also depends on the content of amino acids, the processes applied to the food products, or the production technology used (Ordonez & Callejon, 2020). However, in each tested juice, the total amount of BAs was relatively low (<1 mg/L).

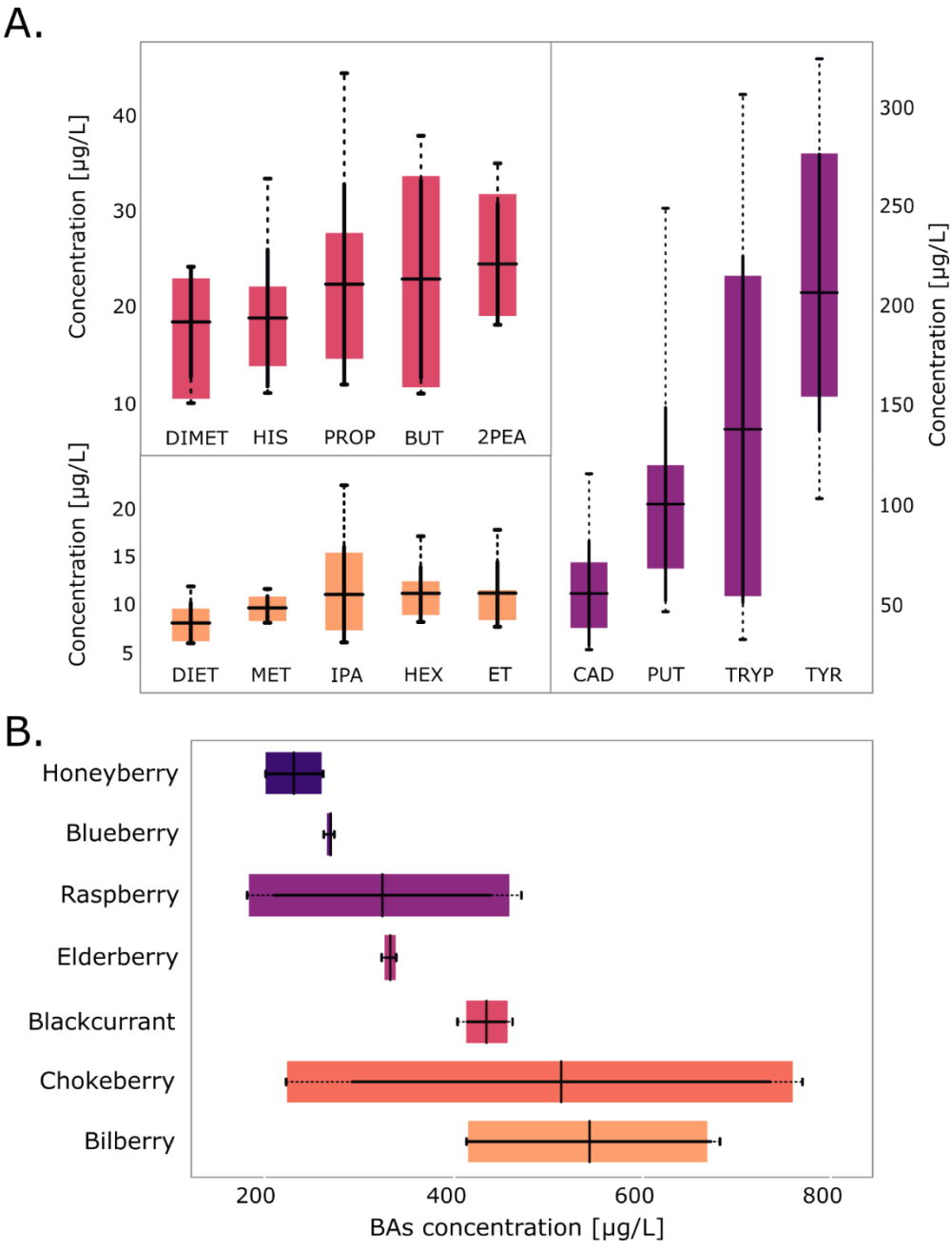

Fig. 4 Biogenic amines concentrations in fruit juices; A - box-plots for biogenic amines, B- box-plots for different juice types

Large variability was observed in the profiles (shown in Fig. 5) of BAs in fruit juices, and even within the same type of juices from different producers. Therefore, a chemometric analysis was performed to check the relationship between each biogenic amine and the berry juices. As shown in Fig. 5, some variables were not relevant for discrimination between juices, while others, e.g. DIMET, PROP, BUT, and CAD, were characteristic for a specific class of samples. Hierarchical clustering of the set of variables revealed that the variables form 2 clusters at h=0.33. The first cluster contained low-concentration BAs and TYR, while the second cluster was comprised of PUT, CAD, HIS, IPA, and 2PEA.

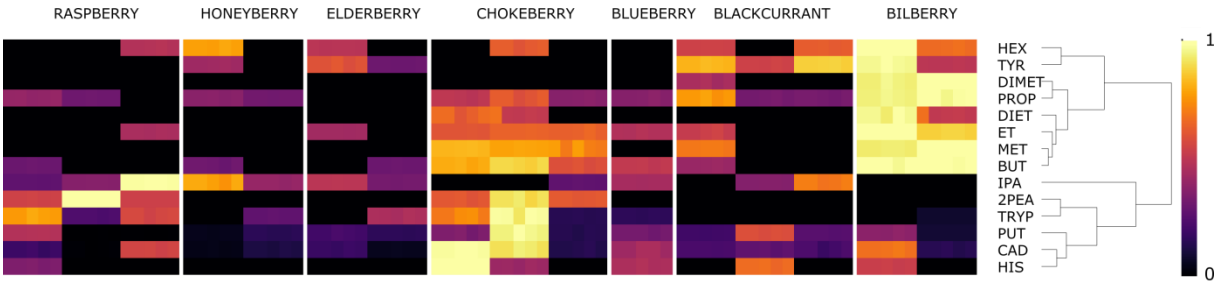

Fig. 5 Heat map depicting the normalized values grouped by class (fruit juice types), with clustered variables

The chemometric approach (e.g. PCA shown in Fig. S2 and S3) confirmed several observations made after preliminary analysis of the GC-MS results. Bilberry juice samples were characterized by a relatively high concentration of DIMET, PROP, ET, MET, and BUT. Based on concentrations of these BAs it was possible to distinguish bilberry juice from other juice types. Relatively high concentrations of 2PEA, TRYP, PUT, and CAD, together with moderately high concentrations of MET and BUT, was typical for chokeberry juice samples. Furthermore, the presence of both 2PEA and IPA at moderately high concentrations was specific for raspberry juice samples. The remaining juice samples have similar BAs profiles. It can be concluded that BAs profiles were characteristic for several juices types and it was possible to distinguished bilberry, chokeberry and raspberry samples from the other berry juices.

4. Conclusions

A new analytical method was developed and fully validated for the simultaneous determination of biogenic amines in fruits juices. The developed method offers the potential of the determination of a high number of compounds (14), combining selectivity, high-resolution capacity and fast analysis time (only 16 min) of GC-MS with the advantages of simple, rapid and reliable extraction procedures. SALLME is a straightforward technique in which small amounts of reagents and solvents are utilized for each extraction. The developed method is inexpensive, reduces the usage of hazardous organic solvents compared to previous approaches, and it is environmentally friendly, which was assessed using two greenness metrics for analytical procedures: GAPI and AGREE. The method has been fully validated and displays satisfactory linearity ($R2 \geq 0.9948$), low LODs (1.5–8.1 µg/L), low LOQs (5.0–26.72 µg/L), excellent accuracy (84–108%), good repeatability (2.6-11.3%) and reproducibility (2.5-11.6%). The obtained results confirmed that the SALLME-GC-MS method was suitable for the determination of BAs at trace levels (µg/L) in liquid food samples. The developed method can be a useful tool for monitoring food quality and ensuring food safety in terms of biogenic amines content.

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