

1 -Green, simple analytical method for biogenic amines determination in fruit 2 juice samples using salting-out assisted liquid-liquid microextraction and gas 3 chromatography-mass spectrometry

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9 **Highlights:**

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

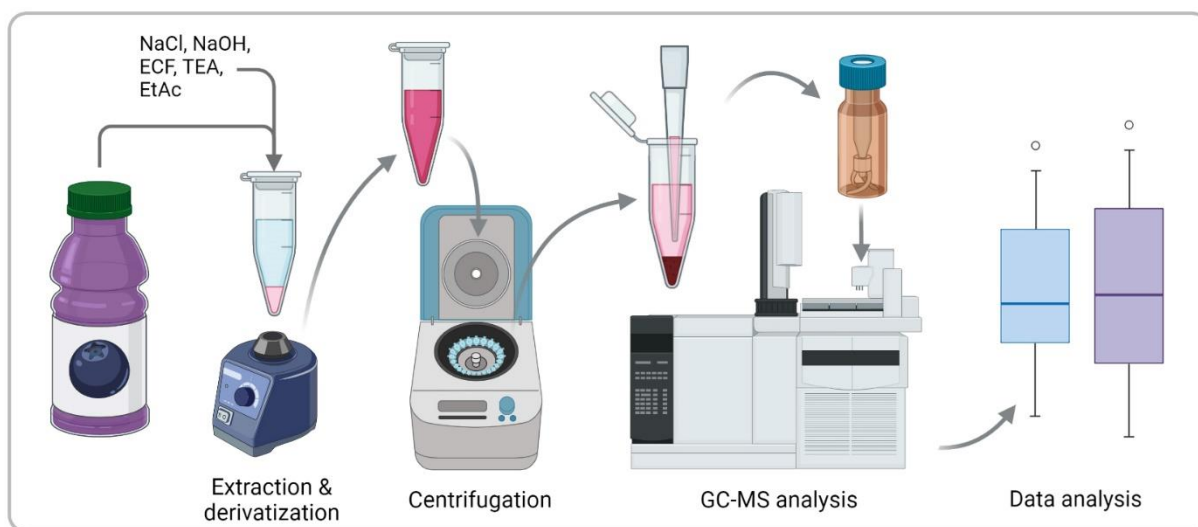
15

16 **Abstract**

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

29

30 Graphical abstract



31

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

34 1. Introduction

35 Biogenic amines (BAs) are nitrogenous organic bases formed in food as a result of the activity
36 of microorganisms capable of decarboxylating amino acids (Wójcik et al., 2021). BAs are commonly
37 found in food products, especially those subjected to fermentation processes (beer, wine), long
38 maturation (cheese), rich in protein (meat, fish), but also fresh fruits and vegetables (Wójcik et al.,
39 2021). Consumption of BAs in low concentrations is not dangerous to the consumers' health.
40 However, consumed in excess, they can cause several toxic effects such as nausea, vomiting,
41 diarrhoea, headache, respiratory failure, and palpitations (Doeun, Davaatseren, & Chung, 2017). In
42 the case of non-fermented beverages, such as e.g. juices, a high BAs content may indicate
43 undesirable activity of microorganisms (Vinci & Maddaloni, 2020). Therefore, BAs concentration can
44 be a useful indicator of food quality and safety (Ruiz-Capillas & Herrero, 2019).

45 Biogenic amines determinations are commonly performed using ion chromatography
46 (Jastrzębska, Piasta, & Szlyk, 2015), liquid chromatography (Eliassen, Reistad, Risoen, & Ronning,
47 2002; Saaid, Saad, Hashim, Mohamed Ali, & Saleh, 2009), and gas chromatography (Cunha, Faria, &
48 Fernandes, 2011; Fernandes & Ferreira, 2000) coupled with various detectors. When developing new
49 analytical methods for the determination of BAs in food, several problems should be bore in mind,
50 namely the low concentration of analytes, the presence of many interfering substances, the complex
51 matrix composition, and the polar nature of BAs. To obtain satisfactory results, the procedure
52 of extraction and derivatization of analytes should be properly selected (Płotka-Wasyłka, Morrison,
53 Biziuk, & Namieśnik, 2015).

54 The most commonly used reagent to derivatize BAs from fruit juice samples is dansyl
55 chloride, since it forms very stable derivatives and can react with both primary and secondary amines
56 (Basheer et al., 2011). The products of such derivatization are then analysed using high-performance
57 liquid chromatography coupled with spectrophotometric or fluorometric detection. Other frequently
58 used BAs derivatizing agents are o-phthalaldehyde (Vieira, Theodoro, & Glória, 2007),
59 1-naphthylisothiocyanate (Jain, Gupta, & Verma, 2015), heptafluorobutyric acid (Fernandes &
60 Ferreira, 2000), and isobutyl chloroformate (Cunha et al., 2011). Alkyl chloroformates are extremely
61 useful in determining BAs using GC-MS because the derivatization step avoids the low sensitivity
62 and the tailing of the peaks due to the high polarity of the amines (Zaikin & Halket, 2003). Moreover,
63 the derivatization process with alkyl chloroformates is inexpensive and less time-consuming
64 compared to derivatization with dansyl chloride. Alkyl chloroformates are reactive in an aqueous
65 medium facilitating their use in the analysis of beverages samples.

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

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

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



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

94 **2. Materials and methods**

95 **2.1. Reagents, chemicals and standards**

96 All the BAs, such as methylamine hydrochloride (MET, 98.0%), dimethylamine hydrochloride
97 (DIMET, 99.0%), ethylamine hydrochloride (ET, 98.0%), diethylamine hydrochloride (DIET, 99.0%),
98 propylamine (PROP, 98.0%), butylamine (BUT, 99.5%), isopentylamine (IPA, 98.0%), hexylamine (HEX,
99 99.0%), 2-phenylethylamine hydrochloride (2PEA, 98.0%), putrescine (PUT, 97.5%), cadaverine (CAD,
100 96.5%), histamine (HIS, 96.5%), tyramine (TYR, 96.5%) and tryptamine (TRYP, 97.5%) were purchased
101 from Merck (Merck Life Science Sp.z.o.o, Poznań, Poland). Aniline (IS, 99.5%), ethyl chloroformate
102 (ECF, 99.5%) and triethylamine (TEA, 99.5%) which were used as internal standard, derivatizing
103 agent and catalyst respectively were also from Merck Life Science Sp.z.o.o (Merck, Poznań, Poland).
104 Ultrapure water for aqueous solutions and glassware washing was prepared using HLP5 Hydrolab
105 (Wiślina, Poland). For the salting-out assisted liquid-liquid microextraction procedure, sodium
106 chloride (NaCl, ACS grade, POCH, Gliwice, Poland), sodium hydroxide (NaOH, ACS grade, POCH,
107 Gliwice, Poland) together with ethyl acetate (EtAc, 99.9%, VWR International, Gdańsk, Poland) were
108 used.

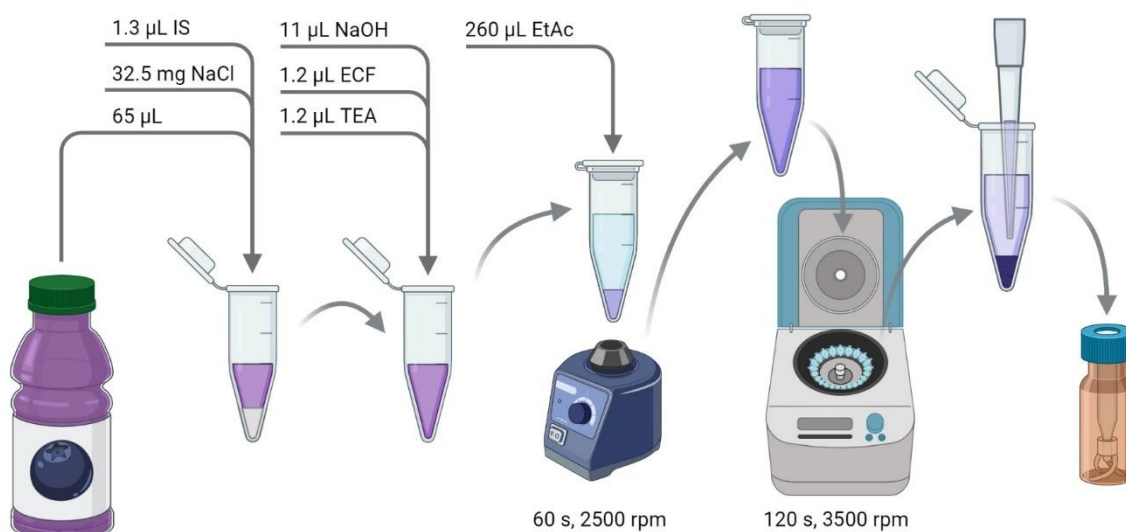
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110 **2.2. Samples and preparation procedure**

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

116 All samples were derivatized according to SALLME protocol. In a 2 mL Eppendorf tube, 32.5
117 mg of sodium chloride and 65 μL of fruit juice (enriched with 1.3 μL of IS at a concentration of 50
118 $\mu\text{g}/\text{mL}$) were mixed. Afterwards, ECF derivatization combined with simultaneous liquid-liquid
119 extraction (LLE) was carried out at room temperature. More in detail, 11 μL of NaOH solution (1.0
120 mol/L) were added to the sample to obtain the pH appropriate for carbamate formation, followed by
121 the addition of 1.2 μL of the derivatizing reagent (ECF) and 1.2 μL of TEA. The solution was mixed
122 with 260 μL of EtAc and then vortexed for 1 min at 2500 rpm. Then, the mixtures in the Eppendorf

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



126
127 *Fig. 1 Scheme of SALLME protocol for BAs analysis in juice samples*

128 2.3. Instrumentation

129 All analyses were carried out on a GC-MS instrument (Agilent Technologies, Santa Clara, CA,
130 USA) consisting of an Agilent 7890A gas chromatograph coupled to an Agilent 5975C single
131 quadrupole mass spectrometer detector. A fused silica capillary column (0.3 m × 0.25 mm,
132 Phenomenex, Torrance, CA, USA) was used as a guard column connected to a ZB-5MS capillary
133 column (0.3 m × 0.25 mm × 0.25 µm, Zebron, Phenomenex, Torrance, CA). Helium (99.999% pure, Air
134 Liquide, Kraków, Poland) was used as a carrier gas with a flow rate of 1.0 mL/min. 2 µL of the extracts
135 were transferred into an injector which was operated in splitless mode at 240 °C.
136 For the chromatographic separation, the GC oven temperature program was as follows: initial
137 temperature 55 °C, held for 4 min, then increased to 280 °C at 50 °C/min and held for 7.5 min. All
138 targeted compounds were separated within 16 min. The total time needed for analysis per sample
139 was 20 min: 1.0 min for reagents preparing, 1.0 min for derivatization and extraction step, 2.0 min
140 for centrifugation and 16.0 min for the chromatographic run. The MS was performed in EI mode (70
141 eV). The transfer line, ion source and detector temperatures were 300 °C, 230 °C and 150 °C,
142 respectively. Synchronous scan/selected ion monitoring (SIM) mode was used for the collection
143 of both types of data in each run (solvent delay: 4.5 min). The scan m/z range was set to 30–500
144 amu. In the SIM mode, one quantification and two qualifier ions were monitored for quantification
145 purposes. Data were acquired using MSD ChemStation, Ver. E.02.00.493 software from Agilent
146

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

149

150 **2.4. Box-Behnken design**

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

167

168 **2.5. Quality assurance (QA)**

169 The optimized method was evaluated using the following validation parameters: linearity,
170 precision, sensitivity and accuracy according to quality assurance protocol. Linearity was examined
171 by application of 10 different concentrations. Limits of detection (LODs) and limits of quantification
172 (LOQs) were calculated to estimate the sensitivity of the methodology. Both LODs and LOQs were
173 calculated from spiked samples ($n=5$) and the minimum detectable analyte amount with a signal-to-
174 noise ratio of 3 and 10, respectively, was established. The intra-day (RSD_r) and inter-day (RSD_R)
175 precision were determined by the application of five replicates of juice samples spiked at two levels
176 (0.25 and 2.5 mg/L). In addition to validation parameters, recovery rates were estimated using
177 the ratio of the peak areas of the spiked samples of known concentration of biogenic amines to those
178 of spiked ethyl acetate solution ($n=5$).

179

180 **2.6. Data analysis**



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

187

188 **3. Results and discussion**

189 **3.1. Analytical method development**

190 **3.1.1. Optimization of SALLME and derivatization protocol**

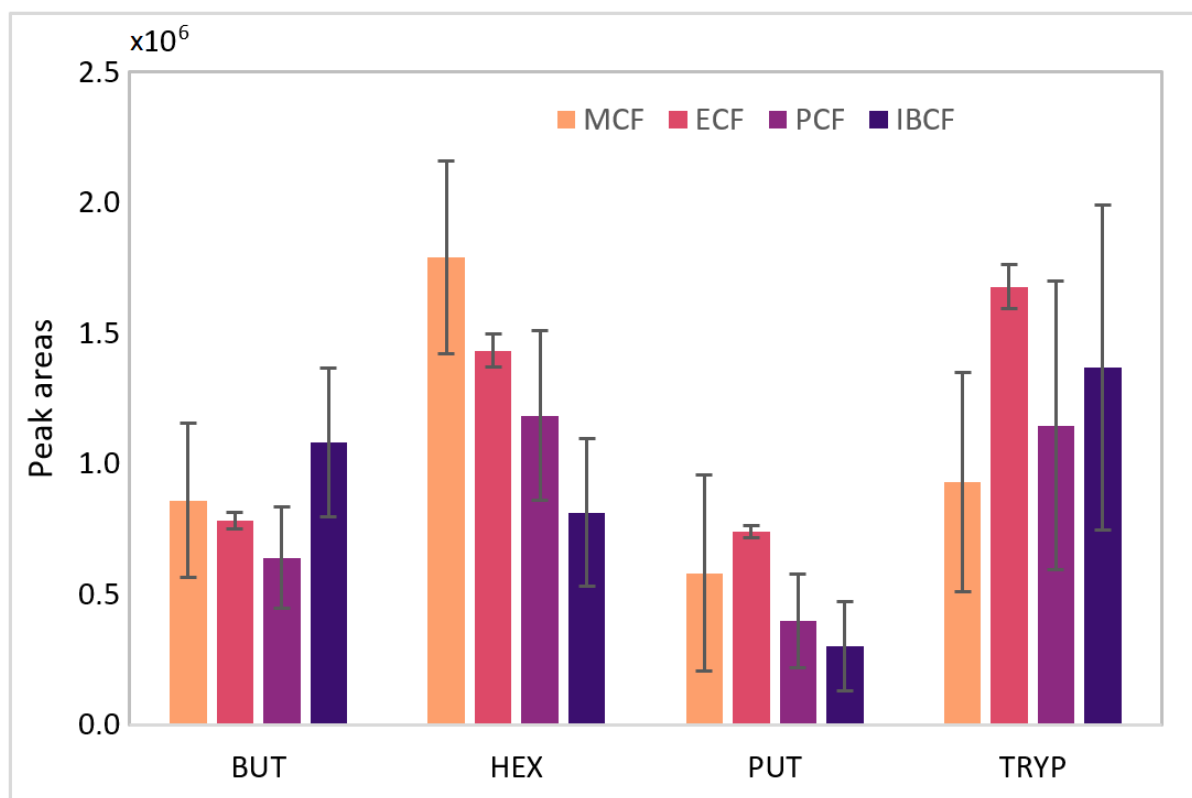
191 The main parameters affecting the efficiency of SALLME are sample volume, type and volume
192 of extractant, duration of extraction, process temperature, and type and amount of added salt (Jain
193 et al., 2015; Ramos et al., 2014). In the proposed procedure, the extraction and derivatization
194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

199 In SALLME, the salting-out effect is used to facilitate the separation of water-miscible organic
200 solvents by adding electrolytes to the solution, which changes the ionic strength and increases
201 extraction efficiency (Francisco et al., 2020; Tsochatzis, Lopes, Gika, Dalgaard, & Theodoridis, 2021).
202 The main factor responsible for the efficiency of phase separation in SALLME procedures is the type
203 of salt anion (Ramos et al., 2020). Another important aspect that needs to be taken under
204 consideration is the fact that the amount of added salt must be sufficient for clear separation
205 of the two phases, but its amount should be as small as possible to avoid adsorption of the analytes
206 on the salt crystals surface. (Tsochatzis et al., 2021). For this reason, the NaCl amount corresponding
207 to 10% w/v of the reaction mixture was chosen. Another crucial factor is the type and volume
208 of the extractant. Ethyl acetate was chosen as the extraction solvent because of its green nature
209 and the fact that it is immiscible both with water and juice samples (Manca et al., 2017; Sánchez,
210 Santos, Sappó, Pavón, & Cordero, 2014). The aim was to develop an extraction procedure performed
211 on a micro-scale, therefore it was decided that the total volume during extraction should not exceed
212 1 mL. Assuming a suitable solvent to sample ratio of 4:1 (Fabio et al., 2020), 200 to 600 μ L of EtAc
213 was used.

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



216 and selectivity of the technique, and improve the quality of the mass spectra. The derivatization
217 process was performed using different alkyl chloroformates, namely methyl chloroformate (MCF),
218 ethyl chloroformate (ECF), propyl chloroformate (PCF), and isobutyl chloroformate (IBCF).
219 The derivatization agent was chosen based on the results obtained during GC-MS analysis of four
220 BAs, selected according to their chemical structure and derivatized with the above-mentioned
221 chloroformates (Fig. 2).
222



223 *Fig. 2 Influence of the derivatization reagent on the resulting peak areas of selected biogenic amines; MCF – methyl*
224 *chloroformate, ECF – ethyl chloroformate, PCF – propyl chloroformate, IBCF – isobutyl chloroformate*
225

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

236 (1mol/L) was added to increase the pH to basic. After adjusting the pH, an optimized volume of ECF
237 was added to the sample. Both parameters, volume of NaOH and ECF were selected for optimization
238 using DoE (Section 3.1.2). TEA was added to the samples to remove the by-product of derivatization,
239 i.e. hydrogen chloride, from the reaction mixture (Husek & Simek, 2006).

240

241 **3.1.2. Response surface methodology for SALLME and derivatization protocols**

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

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

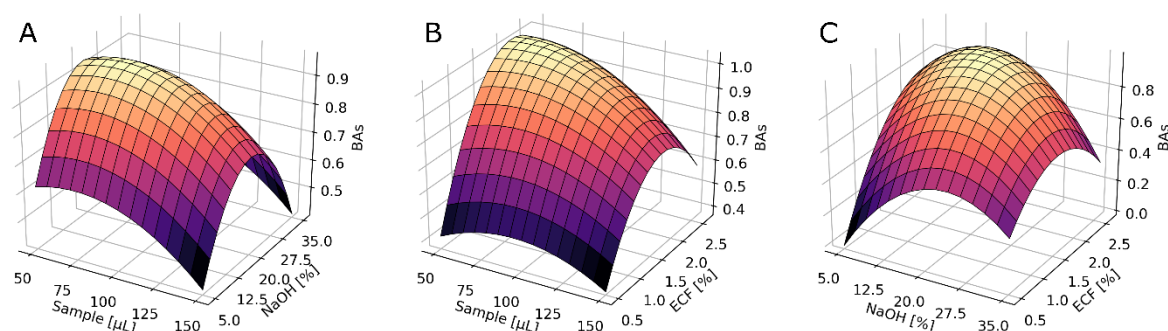
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$$\begin{aligned} 252 \quad BAs = & -1.266 + 0.00806 [Sample] + 0.07552 [NaOH] + 1.332 [ECF] \\ 253 \quad & -0.000039 [Sample]^2 - 0.001473 [NaOH]^2 - 0.2460 [ECF]^2 \\ 254 \quad & +0.000006 [Sample] \cdot [NaOH] - 0.001562 [Sample] \cdot [ECF] \\ 255 \quad & -0.01172 [NaOH] \cdot [ECF] \quad (Equation 1) \end{aligned}$$

256

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

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



271

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

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

292

293 3.1.3. Optimization of GC-MS conditions

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

299 the retention times and MS spectral information with the information obtained from the analysis
300 of standard solutions. It is worth noting that not all BAs bind to a single molecule of ECF. Based on
301 the obtained results, it can be observed that monoamines bind to only one ECF molecule,
302 while polyamines bind to one or two ECF molecules. This information, along with retention times
303 and characteristic fragments obtained, can be found in Table S3. The molecular ion peaks were
304 observed in the mass spectra of all analytes. The m/z 102 fragment appears in the mass spectra
305 of most of the analysed amines. This fragment can be related to the common presence
306 of the CH₃CH₂OC(O)NHCH₂ group in the molecular structures of these compounds, i.e. (N-methyl)-
307 ethyl carbamate group (Reddy, Chary, Pavankumar, & Prabhakar, 2016).

308

309 **3.2. Method validation**

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

$$316 \quad ME [\%] = \frac{B}{A} \cdot 100\% \quad (\text{Equation 2})$$

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

320 The method's linearity and sensitivity were assessed for fourteen BAs by calibration
321 with standard solutions in the presence of IS. Ten EtAc solutions containing all tested BAs in two
322 separate concentration ranges: from 0.05 to 1 mg/L and from 1 to 10 mg/L, respectively, were
323 subjected to the developed procedure. Least squares linear regression was used to calculate
324 the equations of the calibration curves and the determination coefficients (R²). Detailed information
325 for each analyte is provided in Table 1. The linearity was excellent for all analytes with determination
326 coefficients from 0.9948 to 0.9989 (for the first range) and from 0.9956 to 0.9993 (for the second
327 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
328 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	Recovery	Intra-day	Recovery	Day 1	Day 2	Day 3			
	Linearity (R ²)		(%RSD)	(%)	(%RSD)	(%)						
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

331 The intra-day precision (RSD_r) was estimated based on the results of analysis of five replicates
332 of juice samples fortified at two concentration levels (0.25 and 2.5 mg/L) on the same day. The inter-
333 day precision (RSD_R) was determined by analysis of samples from three different days over three
334 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),
335 while RSD_R ranged from 2.5 to 11.6% (Table 1). The stability of the analytes in the juice matrix led
336 to satisfactory precision.

337 The accuracy of the method was determined by a recovery test, i.e. a comparison
338 of the unenriched sample with the samples enriched with analytes at two concentration levels
339 (0.25 and 2.5 mg/L) with five replicates. The recovery rates are listed in Table 1. The average
340 recovery values ranged from 84 to 106% (for 0.25 mg/L) and from 89 to 102% (for 2.5 mg/L). These
341 results indicate that the developed procedure of BAs determination in fruit juices samples was
342 characterized by high accuracy.

343

344 **3.3. Greenness evaluation**

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

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



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

370

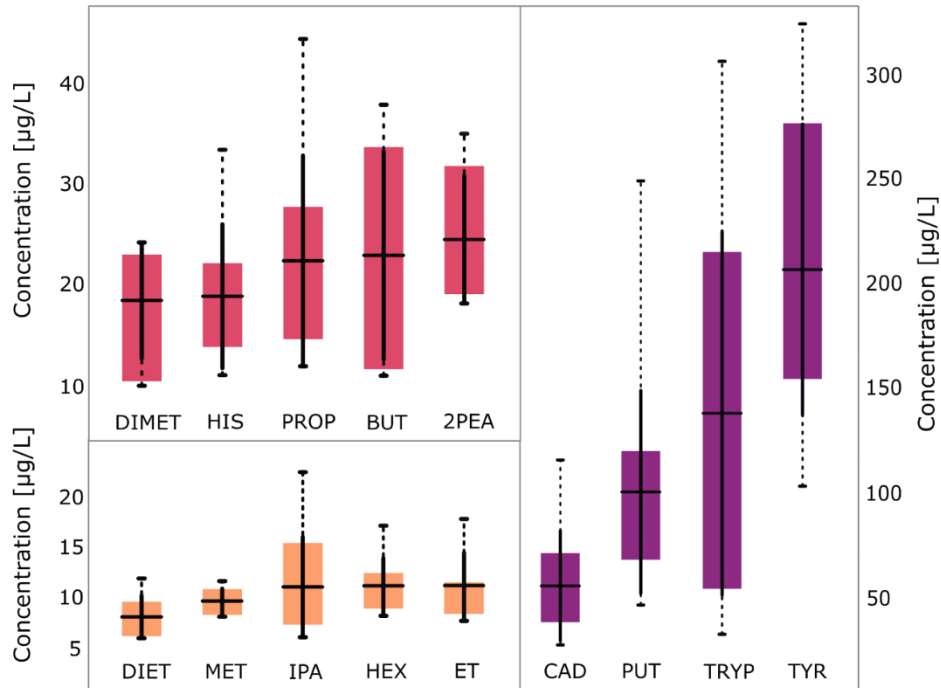
371 **3.4. Real samples analysis**

372 The developed analytical method was used to determine BAs in selected nonfiltered berry juices
373 characterized by a high content of bioactive substances (Table S4). PUT and CAD were detected
374 in each sample which was to be expected since they are typically found in plant-based products
375 (Ordonez & Callejon, 2020). PUT was reported to be the predominant amine in most fruit juice
376 samples (Eliassen et al., 2002). DIMET and DIET were detected only in a few samples. The ranges
377 of each biogenic amine content in berry juices are shown in Fig. 4A. The most abundant amines
378 in fruit juices were TYR with an average concentration of $197 \pm 70 \mu\text{g/L}$, TRYP ($129 \pm 88 \mu\text{g/L}$), PUT
379 ($91 \pm 49 \mu\text{g/L}$), and CAD ($46 \pm 27 \mu\text{g/L}$). The mean concentrations of the remaining BAs ranged from 8.0
380 $\mu\text{g/L}$ for DIET to approx. 25 $\mu\text{g/L}$ for 2PEA. Jastrzębska et al. also noted that the most abundant
381 amines in the samples of non-filtered juices were TYR, PUT, and CAD, and their concentration
382 depended on the type of juice (Jastrzębska et al., 2015). Additionally, Saaid et. al. observed that in
383 tropical fruit juices the most abundant amines were TRYP and HIS, while in blackcurrant juice
384 the most abundant amines were PUT, HIS, and spermidine (Saaid et al., 2009). Low levels of PUT
385 in juices may also suggest that overripe fruit were not used in their production (Jastrzębska et al.,
386 2015). The level of TYR can fluctuate during storage even at refrigerator temperatures and a high
387 content of this amine may indicate that a long time has elapsed between the production of the juice
388 and its purchase (Saaid et al., 2009).

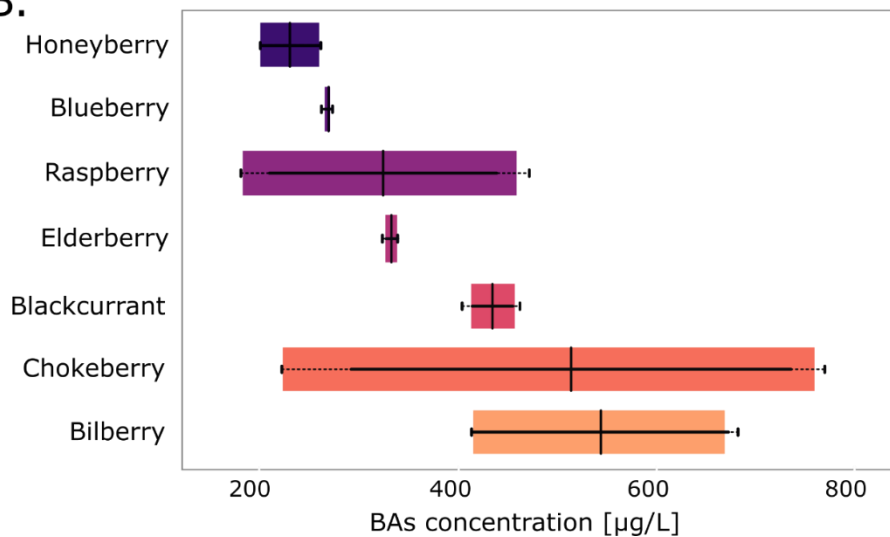
389 The total BAs content depending on the type of juice is shown in Fig. 4B. Based on these
390 results, it can be observed that the samples of honeyberry, blueberry, elderberry, and blackcurrant
391 juices were characterized by a relatively small variation in BAs concentrations. On the other hand,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



A.



B.



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398

399

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

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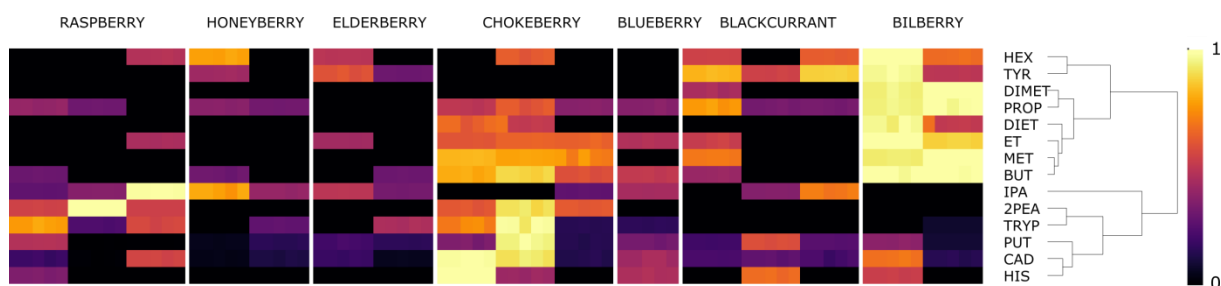
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Large variability was observed in the profiles (shown in Fig. 5) of BAs in fruit juices, and even within the same type of juices from different producers. Therefore, a chemometric analysis was performed to check the relationship between each biogenic 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.



408

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

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

420

421 4. Conclusions

422 A new analytical method was developed and fully validated for the simultaneous
 423 determination of biogenic amines in fruits juices. The developed method offers the potential
 424 of the determination of a high number of compounds (14), combining selectivity, high-resolution
 425 capacity and fast analysis time (only 16 min) of GC-MS with the advantages of simple, rapid
 426 and reliable extraction procedures. SALLME is a straightforward technique in which small amounts
 427 of reagents and solvents are utilized for each extraction. The developed method is inexpensive,
 428 reduces the usage of hazardous organic solvents compared to previous approaches, and it is
 429 environmentally friendly, which was assessed using two greenness metrics for analytical procedures:
 430 GAPI and AGREE. The method has been fully validated and displays satisfactory linearity ($R^2 \geq 0.9948$),
 431 low LODs (1.5–8.1 $\mu\text{g/L}$), low LOQs (5.0–26.72 $\mu\text{g/L}$), excellent accuracy (84–108%), good
 432 repeatability (2.6–11.3%) and reproducibility (2.5–11.6%). The obtained results confirmed
 433 that the SALLME-GC-MS method was suitable for the determination of BAs at trace levels ($\mu\text{g/L}$)
 434 in liquid food samples. The developed method can be a useful tool for monitoring food quality
 435 and ensuring food safety in terms of biogenic amines content.

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444

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