An air-assisted dispersive liquid phase microextraction method based on a hydrophobic magnetic deep eutectic solvent for the extraction and preconcentration of melamine from milk and milk-based products

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ABSTRACT

In the current research, a fast and sustainable air-assisted hydrophobic magnetic deep eutectic solvent-based dispersive liquid phase microextraction followed by UV–Vis spectrophotometry measurements was optimized for the extraction and determination of melamine in milk and milk-based products. The central composite design was applied for the optimization of factors affecting the recovery of melamine. Quantitative extraction of melamine was achieved using hydrophobic magnetic deep eutectic solvents prepared from a mixture of octanoic acid, aliquat-336, and cobalt(II) chloride. The optimum conditions for extraction were found as follows: 6 extraction cycles, pH 8.2, extraction solvent volume 260 μL, and acetone volume 125 μL. Interestingly, a centrifugation step was not required to achieve phase separation. Under the optimum conditions, melamine was determined in the linear range of 3–600 ng mL^{-1}, the limit of detection (3S$_{blank}$/m) of 0.9 ng mL^{-1}, and the enrichment factor of 144. The validation of the method was investigated by the analysis of reference materials. Consequently, the method was successfully applied for the analysis of melamine residues in milk and milk-based products.

1. Introduction

Melamine, as a heterocyclic organic compound, is often used in combination with formaldehyde and other compounds to produce highly durable synthetic resins (Caldara et al., 2022). Kitchen utensils, dinnerware, thermosetting plastics electrical equipment, and adhesive production can be given as examples of melamine applications (Zhu & Kannan, 2019). Melamine has been unfairly used as an additive in foodstuffs to enrich the protein content in foods due to its low cost and high nitrogen-rich content. Misuse of melamine in pet foods was reported in the USA in 2007 while contaminated milk powder was documented in China in 2008. Even though melamine has relatively low toxicity when consumed in its pure form, this latter incident resulted in very negative consequences in terms of the safety of foodstuffs. However, melamine may cause kidney damage in infants and adults depending on the dose and threshold when consumed with other

compounds (such as cyanuric acid) (Skinner, Thomas, & Osterloh, 2010). Excessive intake of melamine can also cause urinary bladder cancer. Furthermore, the absorption of melamine in the bloodstream can even result in death, especially in infants (An et al., 2022). Therefore, the acceptable daily intake of melamine has been recommended by World Health Organization (WHO), suggested as 200 μg/kg bw/day in 2008, which was later reduced to 63 μg/kg bw/day by Food and Drug Administration (FDA) in 2010 (Chen et al., 2021). Due to these negative properties, it is essential to develop new analytical methods for the selective and rapid determination of melamine, especially in milk and milk-based products.

To some extent, several methods have been used for the initial determination of melamine, including Fourier transform infrared spectroscopy (Jawaid et al., 2014), UV–Vis spectrophotometer (Altunay, Elik, & Kaya, 2020), high-performance liquid chromatography (Shirani et al., 2021), capillary electrophoresis (Guo et al., 2020) and gas

chromatography-mass spectrometry (Li, Sun, Li & Xu, 2019). However, as the reliability of the results obtained in the analyses at trace levels decreases due to the matrix effect, an effective and selective sample preparation step should be applied to the selected samples. Over the last decade, several methods have been timely reported for the separation and extraction of melamine in milk and dairy products, including stir bar sorptive dispersive microextraction (Shirani et al., 2021), dispersive liquid–liquid microextraction (Faraji & Adeli, 2017), surfactant-enhanced hollow fiber liquid phase microextraction (Yazdi, Yazdi-nezhad, & Heidari, 2015), ionic liquid-enhanced membrane microextraction (Faustino et al., 2017), electromembrane microextraction (Fashi, Yaftian, & Zamani, 2015), magnetic solid-phase extraction (Abdolmohammad-Zadeh, Zamani, & Shamsi, 2020), matrix solid phase dispersion (Wang, Gao, Qin, & Chen, 2017), among others. Among these mentioned techniques, the most critical factor in sample preparation has been the selection of the right extraction solvent; conventional organic solvents have indeed been the most commonly used. Herein, there is also a need to use more eco-friendly solvents instead of such organic solvents, which are harmful to human beings and the environment.

As eco-friendly alternatives, deep eutectic solvents (DESs) are likely to be one of the most preferred members of the green solvents used in microextraction techniques. DESs are chemically synthesized by combining a hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) at appropriate conditions (Li & Row, 2019). The appropriate combination of such components results in a mixture with a lower melting point than the melting point of each initial component (Augirre & Canals, 2022). Typically, DESs offer several advantages in terms of low heat capacity, high thermal stability, and good biocompatibility. Concurrently, they are easier to prepare, more environmentally friendly, and do not require further purification processes compared with ionic liquids (Peng, Liu, Wang, & Ding, 2021). Considering these properties, they can create a green extraction media for the selective extraction of many analytes (Makoś, Przyjazny, & Boczkaj, 2018). Their high selectivity and multiple interactions with target analytes are other distinctive features (Momotko, Łuczak, Przyjazny, & Boczkaj, 2022; Momotko, Łuczak, Przyjazny, & Boczkaj, 2021). As for their synthesis, quaternary ammonium and phosphonium salts are frequently used as hydrogen bond acceptors (HBA), while alcohols, carboxylic acids, and amines can be given as examples of the most preferred hydrogen bond donors (HBDs) (Pachecho-Fernandez & Pino, 2019).

DESs can be combined with paramagnetic materials to be transformed into a structure that can be affected by an external magnetic field. In this way, magnetic-DES allow a quick and easy collection of analyte-rich extraction phase when used in microextraction techniques while saving processing time (Farooq, Tryon-Tasson, Biswas, & Anderson, 2022). In general, two steps are required for the preparation of magnetic DES. In the first step, HBA and HBD are mixed in the appropriate molar ratio, and then, the resulting mixture is heated at temperatures ranging from 40 °C to 80 °C, obtaining a clear liquid. When the temperature reaches room temperature, the second stage is started, in which a determined metal chloride (such as $FeCl_3$, $CoCl_2$, $MnCl_2$) is added to the obtained DES and subsequently mixed at a certain mole ratio. A low amount of a volatile solvent (such as dichloromethane) is then added to the mixture. After this, the resulting mixture is stirred for about 24 h and evaporated under pressure, the magnetic-DES is finally prepared (Makos-Chełstowska, Kaykhaii, Płotka-Wasylka, & Guardia, 2022).

Recently, researchers have taken advantage of the superior properties of DESs in the analysis of dairy products, including melamine (Shishov, Nizov, & Bulatov 2023; Pochivalov Cherkashina, Shishov, & Bulatov, 2021; Shishov, Terno, Besedovsky, & Bulatov, 2023; Ramezani, Ahmadi, & Absalan, 2020; Qiao et al., 2021). Although the toxicity of DESs is quite low, there are studies in the literature reporting the existence of some conditions that should be considered. For instance, when evaluating DES toxicity on certain bacteria, it was reported that the cytotoxicity of some DES species was higher than the individual toxicity

of its components (e.g., glycerine, choline chloride) (Hayyan et al., 2013). In addition, in another study investigating the toxicity of DESs, it was reported that choline chloride-based DESs had a reducing effect on the life span of hydras, while some DES species had a phytotoxic effect on the growth of *A. sativum* (garlic) roots (Wen et al., 2015). Such studies and evaluations become relevant for obtaining information about the toxicity mechanism of DESs and preventing confusion about whether DESs are toxic or not. Furthermore, such studies shed light on future research in this field, as they reveal methods that can be evaluated by researchers who will study the toxicity of DESs (Marchel, Cieśliński, & Boczkaj, 2022).

This study aims to develop a highly sensitive, fast, simple, robust, and cost-effective sample preparation method for the determination of melamine in milk and milk-based products. As a result of ongoing studies and research, an air-assisted hydrophobic magnetic deep eutectic solvent dispersive liquid phase microextraction (AA-HMDES-DLPME), followed by UV–Vis spectrophotometry measurement, was developed for the simultaneous extraction and determination of melamine contained in milk and milk-based samples. In this method, the HMDES can be easily separated from the aqueous solution with the help of a neodymium magnet. Experimentally, the parameters influencing the extraction efficiency of melamine were optimized in detail using a central composite design. Under optimum extraction conditions, the AA-HMDE-DLPME method presented good analytical features including wide linear range, low LOD, high PF, and good precision/accuracy.

2. Experimental

2.1. Instruments

The pH adjustment of the samples was performed by a digital pH meter (model 630 Metrohm, Switzerland). An ultrasonic bath (SK5210LHC Kudos, Shanghai, China) was used for the preparation of HMDES. Milli-Q water (18.2 MΩ) was obtained from Milli-Direct Q3 system (Millipore, Bedford, MA, USA). Spectrophotometric analysis of melamine was performed using a UV–Vis spectrophotometer (Shimadzu 1800 model, Kyoto, Japan) with 500 μL quartz cells (Fisher, Germany), while a neodymium magnet was used to separate the HMDES layer from the sample matrix.

2.2. Chemicals

First of all, all chemicals used in the current research were obtained from Merck (Darmstadt, Germany). Furthermore, all chemicals were of analytical grade and were used as received without any further purification. Methanol (MeOH), ethanol (EtOH), tetrahydrofuran (THF), acetone, and acetonitrile (ACN) were tested as dispersive solvents. Chemicals, such as 2-octylamine, octanoic acid, thymol, decanoic acid, aliquat-336, trioctylphosphine oxide (TOPO), tetraoctylammonium bromide ($[N_{888}^+]$ $[Br^-]$), trihexyltetradecylphosphonium chloride ($[P_{66614}^+]$ $[Cl^-]$), $CoCl_2$, $MnCl_2$, and dysprosium(II) chloride ($DyCl_2$), were used for the preparation of hydrophobic magnetic deep eutectic solvents. Particularly, citrate, borate, Tris, phthalate and Britton-robinson buffer solutions were used for pH adjustment. As for pH 8.2, Tris buffer solution was prepared by mixing 0.1 M tris (hydroxymethyl) aminomethane (100 mL) and 0.1 M HCl (45.8 mL). A stock solution of melamine (1000 mg L^{-1}) was prepared by dissolving its appropriate amount in acetonitrile–water (1:1, v/v). Prior to the experimental studies, working solutions were prepared by sequential dilution of the stock solution.

2.3. Sample collection and pre-treatment

Milk and milk-based samples were obtained from markets in Sivas, Turkey. Raw milk, cow milk, and yogurt samples were collected monthly from local producers between October 2022 and February

2023. Other milk-based samples, including ready baby food, flavored milk, ultra-high temperature (UHT) milk, milk powder, fruit yogurt, strawberry milk, and cacao milk, were collected from local markets between November 2022 and January 2023. The following sample preparation procedure was applied to the collected samples before the extraction step (Altunay et al., 2020). Initially, solid dairy products (2 g) and milk (4 mL) were carefully added to the beakers including 1.5 mL of 300 g L^{-1} of trichloroacetic acid. Then, the beaker was vortexed at 3200 rpm for 2 min. After this step, ACN (7.5 mL) was added to the resulting mixture, and the beakers were placed in an ultrasonic bath and sonicated for 10 min at 30 °C. After the centrifugation step (4000 rpm for 5 min), the resulting mixture was filtered and made ready for the application of the AA-HMDE-DLPME method. All studies were performed in parallel with both sample blank and standard melamine-enriched samples.

2.4. Preparation of hydrophobic magnetic deep eutectic solvents

In the current research, hydrophobic magnetic deep eutectic solvents (HMDES) were prepared according to the method previously reported in the literature (Farooq, Ocaña-Rios, & Anderson, 2022). The experimental steps are explained below. First, five different HMDES were prepared by dissolving the components in methanol, which was later removed. Appropriate amounts of hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), and metal halides (forming HMDES) were weighed on an analytical balance and were later transferred to beakers containing 10 mL of methanol. Afterward, the beakers were placed on the magnetic stirrer plate and mixed at 1200 rpm until a homogeneous solution was obtained. Finally, a rotary evaporator was used to remove the methanol. The molar ratios of HBA, transition metal halide, and HBD for the prepared HMDESs are presented in Table 1.

2.5. Experimental design

The central composite design (CCD) was implemented to optimize the important extraction parameters affecting the recovery of melamine. A four-factor five-level CCD was applied to optimize the different extraction parameters, such as extraction cycle (2–10), HMDES-5 vol (110–890 μL), pH (4.25–10.75), and acetone volume (70–330 μL). A total of 30 experiments were conducted including 6 central experiments. The variables of the established CCD model, their symbols, and units are shown in Table S1 (Supplementary File). After optimization, the effect of the parameters on the recovery of melamine was adjusted according to the following equation (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y: response; X_1, X_2 and X_3: variables; β_1, β_2, and β_3: linear coefficients; β_{12}, β_{13}, and β_{23}: coefficients of binary interaction; β_{11}, β_{22}, and β_{33}: quadratic coefficients; β_0: a constant.

2.6. AA-HMDE-DLPME method

The experimental procedure of the AA-HMDE-DLPME method is summarized below. First, a sample solution (5 mL) was added to the centrifuge tubes including 20 ng mL^{-1} of melamine. Then, the pH of the solution was adjusted to pH 8.2 using a 0.1 M Tris buffer solution. For separation and preconcentration of melamine from the sample solution, HMDES-5 (a mixture of octanoic acid, aliquat-336, and CoCl$_2$ at a 3:2:1 M ratio) (260 μL) and acetone (125 μL) were added to the tubes using a micro-syringe. Then, the resultant mixture in the centrifuge tube was withdrawn using the syringe and injected back into the tubes. When this step was repeated 6 times, a turbid mixture was obtained. At this stage, the mass transfer of the melamine in the sample solution to the HMDES phase was achieved. Subsequently, a neodymium magnet was placed at the bottom of the tube, and the HMDES phase along with the targeted analyte was collected at the bottom. The liquid phase was evacuated and the final volume of the HMDES phase was diluted using acetone to a final volume of up to 1 mL. Then, the final solution was placed in the beam path of UV–Vis spectrophotometry, and absorbance measurements were performed at 291 nm (see Fig. S1). The experimental steps described above were also run in triplicate with the sample blank.

2.7. Calculations of matrix effect and recovery

The following equations (2–4) were used to calculate the matrix effect, extraction efficiency, and recovery parameters.

$$\text{Matrix effect} = \left(\frac{X - Y}{Z} - 1 \right) x100 \quad (2)$$

$$\text{Extraction efficiency} = \left(\frac{T - Y}{X - Y} \right) x100 \quad (3)$$

$$\text{Recovery} = \left(\frac{T - Y}{Z} \right) x100 \quad (4)$$

where X is the amount of the melamine for the sample spiked with the melamine after the AA-HMDE-DLPME method, and Y is the amount of the melamine for a non-spiked sample. However, as no milk samples were present in the blank samples, the amount of Y was omitted, Z is the amount of the melamine for the standard solution, while T is the amount of the melamine for the sample spiked with the melamine before the AA-HMDE-DLPME method.

3. Results And discussion

3.1. Selection of the appropriate HMDES and the effect of its molar ratio

The type of extraction solvent is crucial for the selective and quantitative analysis of melamine. In this regard, five HMDESs were prepared and tested for efficient separation of melamine from the sample solution. All HMDESs were used in equal volumes (400 μL). In addition, the AA-HMDE-DLPME method was applied with HMDES solutions prepared in mole ratio of 2:2:1 (HMDES-1), 3:3:1 (HMDES-2), 3:3:1(HMDES-3), 3:3:1(HMDES-4) and 3:2:1(HMDES-5). The experiments were carried out in triplicate. As shown in Fig. 1a, the best extraction efficiencies for melamine were HMDES-5 (94.2%), HMDES-1 (84.6%), HMDES-3 (76.2%), HMDES-4 (70.9%), and HMDES-2 (65.2%), respectively. Here, the best analytical results were obtained with hydrophobic magnetic DES prepared by mixing octanoic acid, aliquat-336 and CoCl$_2$. It is

Table 1
HBA, metal halide, HBD abbreviations, relative molar ratios, physical appearances and viscosities used in the preparation of the tested HMDES.

Symbol	HBD	HBA	Metal halide	Molar ratio	Viscosity (cP)	Appearance at 25 °C
HMDES-1	2-octylamine	TOPO	CoCl$_2$	2:2:1	655	Light yellow liquid
HMDES-2	Octanoic acid	[N$_{888}^+$] [Br$^-$]	MnCl$_2$	3:3:1	4334	Clear liquid
HMDES-3	Thymol	TOPO	CoCl$_2$	3:3:1	247	Light yellow liquid
HMDES-4	Decanoic acid	[P$_{66614}^+$][Cl$^-$]	DyCl$_2$	3:3:1	4764	Clear liquid
HMDES-5	Octanoic acid	Aliquat-336	CoCl$_2$	3:2:1	428	Light yellow liquid

TOPO: Trioctylphosphine oxide, [N$_{888}^+$] [Br$^-$]:Tetraoctylammonium bromide, [P$_{66614}^+$][Cl$^-$]: Trihexyltetradecylphosphonium chloride.
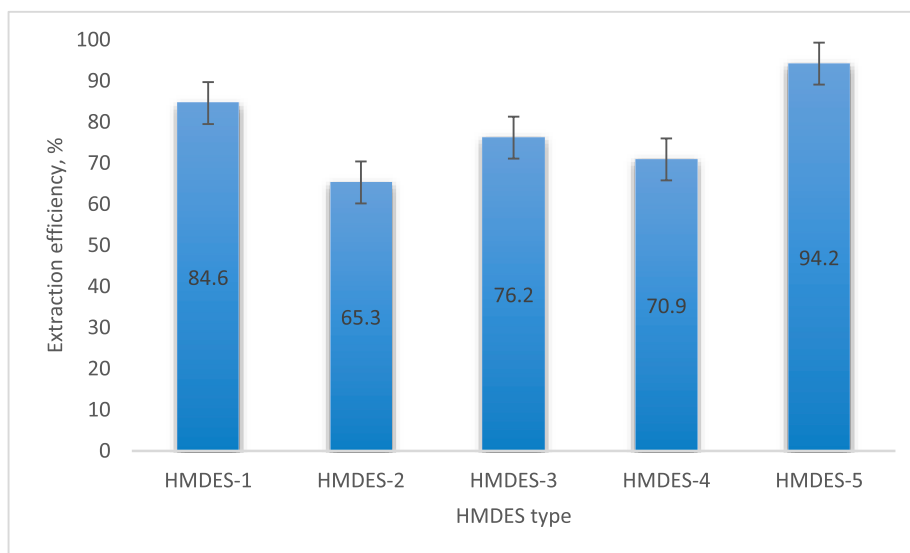
Fig. 1a. Effect of HMDES type on the extraction efficiency of melamine (N = 3).

likely that the use of aliquat-336, which displays a high ionic character in the formation of DES, contributed to obtaining these results. Thanks to aliquat-336, H-bond formation was more effective than other types. Thus, the HMDES-5 prepared from a mixture of octanoic acid, aliquat-336, and $CoCl_2$ was chosen as the extracting solvent in subsequent studies.

Before the CCD optimization step, it was necessary to select the right molar ratio of the extracting solvent, as the effectiveness of HMDES-5 greatly depends on its molar ratio. Therefore, the efficiency of the extraction step may change. Based on these facts, the effect of the molar ratio of components of HMDES-5 on the recovery of melamine was investigated. The extraction efficiency performed at different molar ratios is presented in Fig. 1b. In particular, the extraction efficiency of melamine decreased as the molar ratio of octanoic acid decreased in HMDES-5. This can be ascribed to the incomplete H-bond formation since the amount of hydrogen bond donor in the aqueous solution was insufficient. Interestingly, the best separation according to the quantitative extraction efficiency of melamine was obtained at the molar ratio 3:2:1 for HMDES-5. Thereby, this molar ratio (3:2:1) was chosen as the appropriate molar ratio for HMDES-5 in subsequent studies.

3.2. Selection of dispersive solvent type

In DLPME studies, the type of dispersive solvent is important to increase the effective dispersion of the extraction solvent in the sample solution. The dispersive solvent must be dispersed in both the sample solution and extraction solvent. In this way, the mass transfer of the target analyte is accelerated from the sample solution to the extraction solvent. In light of these explanations, the effect of dispersive solvents, such as EtOH, MeOH, acetone, ACN, and THF, on the extraction efficiency of melamine has been investigated (see Fig. 1c). Compared to other dispersive solvents, acetone provides high extraction efficiency, since it provides effective dispersion in both aqueous solution and the HMDES-5. Based on the results, acetone was chosen as the dispersive solvent in subsequent studies.

3.3. Effect of NaCl amount on extraction efficiency of melamine

In DES-based microextraction studies, different salt solutions (based on NaCl, KCl, KNO_3) are sometimes added to the sample solution to enhance easy separation of the DES phase containing analyte from the
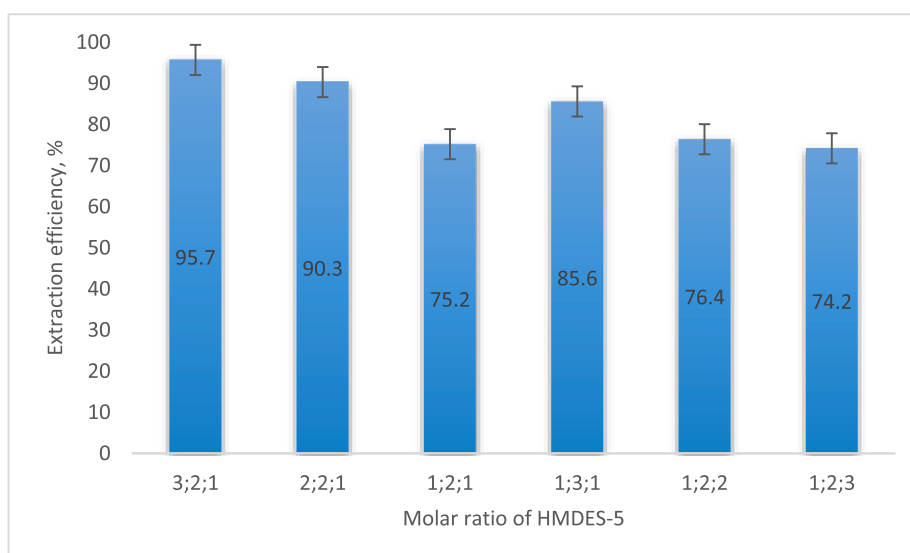

Fig. 1b. Effect of molar ratio of HMDES-5 on the extraction efficiency of melamine (N = 3).
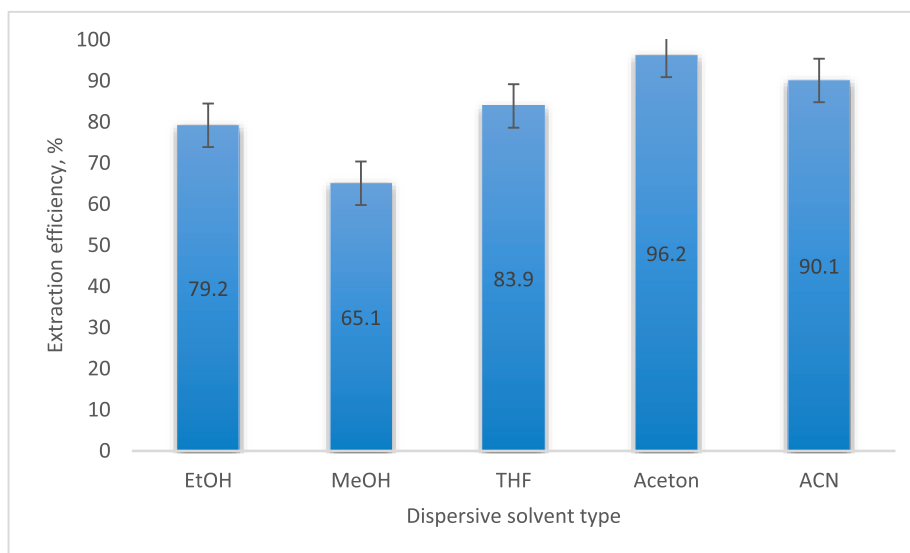
Fig. 1c. Effect of dispersive solvent type on the extraction efficiency of melamine (N = 3).

sample solution. This phenomenon is so-called the salting-out effect. To evaluate this effect, different concentrations of NaCl solution were added to the sample solution, and the extraction efficiency of melamine was then calculated by applying the AA-HMDE-DLPME method. As shown in Fig. 1d, the extraction efficiency did not change with the addition of NaCl solution. For this reason, NaCl solution was not used in subsequent studies.

3.4. Effect of sample volume on extraction efficiency of melamine

The sample volume should be investigated for the calculation of the preconcentration factor (PF) of the AA-HMDE-DLPME method, and the determination of the sample volume from which quantitative recovery was obtained. The PF was calculated from the ratio of the sample volume to the final volume obtained after AA-HMDE-DLPME method. The effect of sample volume on the extraction efficiency of melamine was investigated ranging from 5 mL to 240 mL. The extraction efficiency of melamine was quantitative in sample volumes between 5 and 160 mL (see Fig. 1e). However, at higher sample volumes, there was a drastic decrease in the extraction efficiency. Based on this result, the sample

volume was chosen as 160 mL while the PF was calculated as 160.

3.5. Optimization of the extraction conditions by CCD

The recovery of melamine obtained from each experiment, as planned by CCD, is presented in Table S2. Statistical analysis of the results has been done with one-way analysis of variance (ANOVA). The main components in ANOVA were explained in detail in Table 2. The first parameter evaluated was the significance of the established CCD model for the extraction of melamine. For the CCD to be significant, the p-value must be less than 0.05 at 95% confidence level. Also, the p-value must still be less than 0.05 for each parameter to contribute to the CCD. Based on this, the CCD was significant because its p-value was <0.0001. In addition, linear, binary, and quadratic interactions were significant except for acetone volume (p = 0.4195), extraction cycle* HMDES-5 vol (p =), and extraction cycle* acetone volume (p = 0.1319) interactions. The F-value of the interactions was directly proportional to their contribution to the CCD. According to this explanation, the linear, binary, and quadratic interactions that mostly contributed to the CCD were HMDES-5 vol (F = 422.68), pH*acetone volume (F = 564.53), and
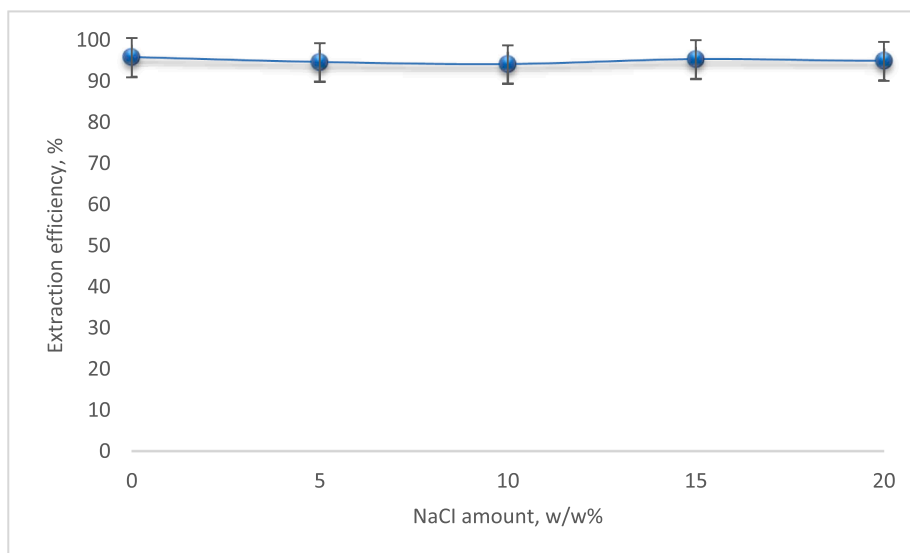

Fig. 1d. Effect of NaCI amount on the extraction efficiency of melamine (N = 3).
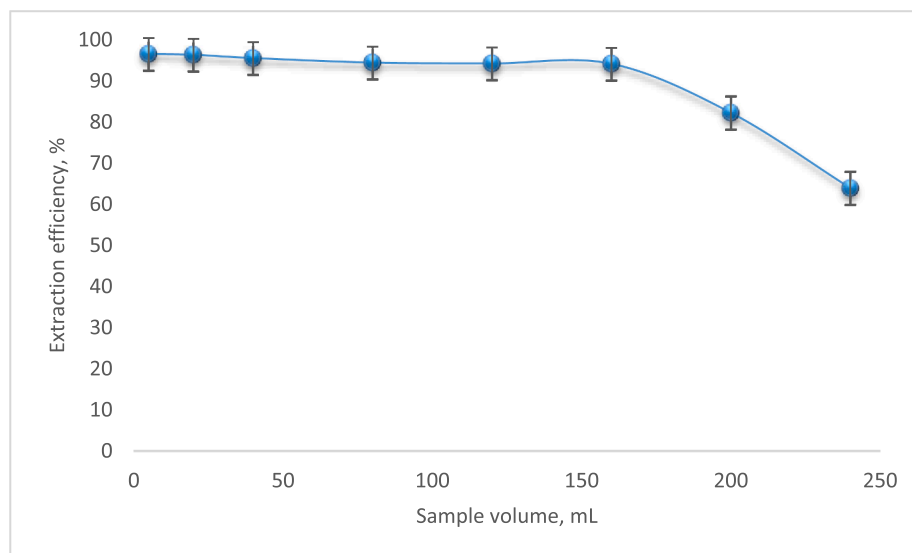
Fig. 1e. Effect of sample volume on the extraction efficiency of melamine (N = 3).

Table 2
Regression coefficients values estimated for extraction of melamine.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1989.46	14	142.10	285.08	< 0.0001	significant
A	210.69	1	210.69	422.68	< 0.0001	significant
B	89.81	1	89.81	180.18	< 0.0001	significant
C	7.31	1	7.31	14.66	0.0016	significant
D	0.3435	1	0.3435	0.6890	0.4195	not significant
AB	47.96	1	47.96	96.21	< 0.0001	significant
AC	0.1406	1	0.1406	0.2821	0.6031	not significant
AD	1.27	1	1.27	2.54	0.1319	not significant
BC	230.28	1	230.28	461.98	< 0.0001	significant
BD	281.40	1	281.40	564.53	< 0.0001	significant
CD	90.73	1	90.73	182.01	< 0.0001	significant
A^2	3.04	1	3.04	6.10	0.0261	significant
B^2	555.60	1	555.60	1114.61	< 0.0001	significant
C^2	516.90	1	516.90	1036.98	< 0.0001	significant
D^2	128.91	1	128.91	258.61	< 0.0001	significant
Quality parameters						
R^2		0.9963	Predicted R^2		0.9816	
Adjusted R^2		0.9928	Adeq Precision		66.6959	

pH*pH (F = 1114.61), respectively. The quality parameters of CCD were R^2, predicted-R^2, adjusted R^2, and adequate precision. Here, the high agreement of the experimental results with the predicted values was investigated with the R^2 values. As the agreement between the experimental and the predicted results of the CCD increases, the R^2 gets closer to 1. The R^2 (0.9963) from the results shows an exceptional agreement between the experimental and predicted values (see Table 2). In addition, the regression between the experimental and the predicted values of CCD is presented in Fig. S2a. In addition, adequate precision was used to evaluate the effect of the signal-to-noise ratio on the CCD. For the CCD to be statistically significant, the adequate precision must be greater than 4. As reported in Table 2c, the results demonstrated that the adequate precision obtained (66.6959) was much larger than the critical value. In another statistical evaluation, the difference between predicted R^2 and adjusted R^2 must be less than 0.2 for CCD to be significant. Therefore, the results obtained support such explanation. Briefly, all evaluations in the ANOVA analysis showed that the CCD was significant. As a result, the CCD model successfully adapted the effect of optimized factors on the recovery of melamine according to the following quadratic equation (5).

$$Recovery(\%) = +80.43 - 3.30A + 2.15B - 0.6140C + 0.1331D + 1.73AB$$
$$+ 0.0938AC + 0.281AD - 3.79BC + 4.19BD + 2.38CD$$
$$- 0.6615A^2 - 8.95B^2 + 8.63C^2 + 4.31D^2$$

$$(5)$$

Response surface methodology was used to plot the effect of binary interactions of optimized factors on the recovery of melamine. The effect of the extraction cycle versus HMDES-5 volume on the recovery of melamine is shown in Fig. S2b. It can bee seen that acceptable recoveries were achieved at low and high HMDES-5 volume, especially when the extraction cycles were less than 8. This could be attributed to the mass transfer of HMDES-5 to the sample solution, which was achieved with less number of extraction cycles. Moreover, it can be attributed to the high selectivity of the prepared HMDES-5 for melamine. The effect of pH versus HMDES-5 volume on the recovery of melamine is given in Fig. S2c. To some extent, the recovery of melamine was quantitative when pH and DES-2 volumes were in the range of 6.8–9.2 and 120–380 μL, respectively. Interestingly, phase separation could not be achieved at low pH due to excessive protonation of HMDES-5 in the acidic region. The effect of acetone volume versus HMDES-5 volume on the recovery of melamine was shown in Fig. S2d. Here, acetone, as a dispersive solvent, helped to increase its interaction with melamine by effectively

dispersing HMDES-5 in the sample solution. In this way, the melamine in the sample solution was easily transferred to the HMDES- phase. Due to this phenomenon, quantitative recoveries were obtained when acetone volume and HMDES-5 volumes were in the range of 90–180 µL and 120–350 µL, respectively.

In the optimization step, CCD was applied to maximize the recovery of melamine. According to the CCD model, the maximum recovery is obtained when the extraction cycle, pH, HMDES volume, and acetone volume are 6, 8.2, 260 µL, and 125 µL, respectively. After five replicates, the experimental recovery of melamine was as high as 91.7%, which agrees with the predicted value (92.4%) of the CCD model. Therefore, these extraction conditions were chosen as optimum for the method validation and analysis.

3.6. Analytical figures of merit for the developed AA-HMDE-DLPME method

The analytical figures of the developed AA-HMDE-DLPME method for the determination and extraction of melamine were calculated using the optimized conditions (6 extraction cycles, pH of 8.2, 260 µL of HMDES-5 vol, and 125 µL of acetone volume). The obtained results are given in Table S3. The regression equation of the method was $A = (8.9 \pm 0.1) \times 10^{-3}$ [melamine, ng mL^{-1}] - $(3.6 \pm 0.9) \times 10^{-4}$ with 0.9976 of the regression coefficient. The detection limit (LOD, 0.9 ng mL^{-1}) and quantification limit (LOQ, 3 ng mL^{-1}) of the AA-HMDE-DLPME method were calculated from formulas $3S_{blank}/m$ and $10S_{blank}/m$, respectively. The S_{blank} was the standard deviation of the eight replicate results of the sample blank, while m was the slope of the calibration plot. The linear range of the method ranged from 3 ng mL^{-1} to 600 ng mL^{-1} with an enrichment factor of 144. Importantly, the enrichment factor was calculated from the ratio of the slopes of the calibration graphs obtained before and after the AA-HMDE-DLPME method. The relative standard deviation (RSD%) and extraction recovery for 20 ng mL^{-1} of melamine (N = 6) were 1.3% and 97.9%, respectively.

3.7. Evaluation of the matrix effect on the developed method

The most important constraint in the analysis of real samples is the matrix effect. To minimize this latter effect, the developed method should exhibit high selectivity for the target analyte. Since the optimization step is carried out with studies on model solutions, there is no matrix effect. In light of these facts, the matrix effect of the AA-HMDE-DLPME method was investigated by applying the experimental procedure described below. First, the chemical species in Table 3 were added to the model solutions at different rates. Then, by applying the AA-HMDE-DLPME method to the obtained mixture, the recovery and RSD % of melamine were calculated in the presence of chemical species. In addition, the tolerable limit was calculated for each chemical specie. As reported in Table 3, it can be seen that the AA-HMDE-DLPME method provides quantitative recoveries ranging from 94 ± 6 to $99 \pm 3\%$ while showing low RSD ($\leq 2.8\%$) in the presence of different chemical species. The tolerable limit was calculated from the ratio of the chemical species concentration to the added melamine concentration, causing a 5% change in the melamine analytical signal. The high tolerable limits proved that the AA-HMDE-DLPME method has a low matrix effect. Preliminary, it can be concluded that the AA-HMDE-DLPME method can be successfully applied to the analysis of melamine in the presence of different chemical species.

3.8. Precision and accuracy of the developed method

The accuracy and precision of the method were investigated by performing intraday and interday studies for three different concentrations (10, 300, and 500 ng mL^{-1}) of melamine. Comprehensive data is presented in Table S4. An intraday study, for added melamine concentrations on the same day, was carried out with five repetitions of the AA-

Table 3
Selectivity of the AA-HMDE-DLPME method for 100 ng mL^{-1} of melamine in the presence of different ions and molecules.

Ions and molecules	Recovery (%)	RSD (%)	*Tolerable limit
Ca^{2+}	99 ± 1	2.0	7500
K^+	97 ± 3	1.9	7500
SO_4^{2-}	98 ± 2	1.5	5000
CO_3^{2-}	97 ± 4	1.8	5000
Mg^{2+}	99 ± 3	1.6	3000
F^-	97 ± 4	1.9	3000
Cl^-	97 ± 2	1.7	3000
Lactose	98 ± 5	1.5	1000
Glucose	96 ± 3	2.0	1000
Zn^{2+}	97 ± 2	1.6	1000
Fe^{3+}	98 ± 2	1.9	750
Biotin	97 ± 4	2.3	750
Pantothenic acid	97 ± 3	2.2	750
Cyromazine,	97 ± 4	2.0	250
Ergocalciferol	97 ± 5	2.1	250
Cobalamin	96 ± 3	2.4	250
Histamine	96 ± 6	2.8	250
Cyanuric	96 ± 2	2.4	100
Methionine	95 ± 4	2.8	100
Ammelide	94 ± 3	2.9	100
Thiamine	95 ± 5	2.6	100
Mannitol	94 ± 6	2.9	100

* [Ion or molecule concentration, ng mL^{-1}] / [melamine concentration, ng mL^{-1}].

HMDE-DLPME method. Herein, the RSD for 10, 300, and 500 ng mL^{-1} of melamine concentrations was 3.1%, 2.9%, and 3.4%, respectively. As for the interday study, this was performed with five replicates for added melamine concentrations on three consecutive days. The RSD for 10, 300, and 500 ng mL^{-1} of melamine were 3.5%, 3.8%, and 4.2%, respectively. In both studies, the recoveries for the added melamine concentrations were in the range of 90.3–97.7%.

The accuracy of the AA-HMDE-DLPME method was tested by analysis of reference material containing milk powder TFV026RM. As shown in Table 4a, the amount of melamine in the reference material was found to be 11.9 ± 0.9 mg kg^{-1}. This value found was consistent with the reference value (12.4 ± 0.7 mg kg^{-1}) at the 95% confidence level (N = 5). The t-exp (1.24) was less than t-critical (2.13), confirming the statistical validity of the method. The obtained quantitative recovery (95.9%) also showed that the AA-HMDE-DLPME method exhibits high accuracy.

3.9. Application of the method to milk-based products

Following validation studies, the applicability of the AA-HMDE-DLPME method was investigated by analyzing different real milk-based samples, such as yogurt, fruit yogurt, strawberry milk, cacao milk, milk powder, UHT milk, raw milk, cow milk, ready baby food, and flavored milk. For the reliability of the results, the selected samples were also analyzed with a reference method (Rambla-Alegre et al., 2010), while the statistical evaluation of the results for both methods was made by calculating t-exp. The results obtained are given in Table 4b. For instance, the t-exp for all samples was smaller than the t-critical. This confirmed that the results obtained with the reference method and the AA-HMDE-DLPME method were not statistically different. The lowest

Table 4a
Application of the AA-HMDE-DLPME method to the reference material for the accuracy of the method (N = 5).

Reference material	Reference, value mg kg^{-1}	Calculated value, mg kg^{-1}	Recovery (%)	t-test
Milk Powder TFV026RM	12.4 ± 0.7	11.9 ± 0.9	95.9	1.24

Table 4b
Application of the AA-HMDE-DLPME method for analysis of melamine in the collected samples.

Samples	Added, ng mL^{-1}	RSD (%)	Recovery (%)	Found (ng mL^{-1})	Reference method	Matrix effect (%)	*t-test
Yogurt	80	1.1	96.4	77.1	78.2	6.2	0.69
	150	1.4	97.9	146.9	145.3	9.3	0.85
Fruit yogurt	80	1.3	95.1	76.1	76.9	3.4	0.79
	150	1.6	96.7	145.0	145.8	4.6	0.62
Strawberry milk	80	1.9	91.2	72.9	71.3	2.8	1.14
	150	1.5	94.6	141.9	143.2	5.6	1.02
Cacao milk	80	1.9	94.3	75.4	74.8	5.9	1.33
	150	2.2	97.1	145.6	144.9	4.4	1.21
Milk powder	80	1.3	96.8	77.4	76.3	10.1	0.96
	150	1.4	97.9	146.8	148.6	7.2	1.08
UHT milk-1	80	1.2	93.4	74.7	75.5	3.5	1.34
	150	1.7	97.6	146.4	148.2	4.9	1.26
UHT milk-2	80	1.8	93.7	74.9	73.7	3.7	0.59
	150	2.0	96.1	144.1	145.3	4.6	0.62
UHT milk-3	80	1.9	92.2	73.8	71.9	5.3	0.78
	150	2.3	95.8	143.7	143.9	4.1	0.83
Raw milk-1	80	1.4	93.3	74.6	75.8	6.6	0.92
	150	1.6	97.9	146.8	147.6	6.2	1.11
Raw milk-2	80	1.7	91.8	73.4	74.4	7.3	0.96
	150	2.1	97.6	146.4	149.3	7.2	1.08
Raw milk-3	80	1.6	93.9	75.1	74.2	5.8	0.88
	150	1.9	96.4	144.6	146.8	6.4	0.96
Cow milk	80	1.3	90.6	72.5	73.6	9.5	1.17
	150	1.5	94.3	141.5	144.0	10.6	1.35
Ready baby food	80	1.4	97.7	78.2	79.2	2.8	0.73
	150	1.9	98.3	147.5	149.1	4.1	0.86
Flavored milk	80	1.3	95.1	47.5	48.6	5.5	1.02
	150	1.8	96.7	145.0	146.2	6.8	1.18

*Based on the statistical comparison of the two mean values obtained by two calibrations approaches, in which the tabulated *t*-value is 2.13 for the degree of freedom of 4 at 95% confidence level.

and highest RSD values for the samples were 1.1% (yogurt) and 2.3% (UHT milk-3), respectively. The lowest and highest recoveries for the samples were 90.6% (Cow milk) and 98.3% (ready baby food), respectively. All these results indicated that the AA-HMDE-DLPME method is reliable to apply for milk and milk-based samples.

3.10 . Comparison with previous studies

The results obtained with our AA-HMDE-DLPME method were compared with similar methods. Herein, such methods were evaluated in terms of linear range, LOD, PF, RSD, and extraction time. The comparison among methods is presented in Table S5, basically, the AA-HMDE-DLPME method required the shortest extraction time (3 min), lowest RSD (1.3%), and highest PF (160) among the reported methods. The LOD (0.9 ng mL^{-1}) of this method was lower than most of the reported methods. However, it is worth mentioning that specific methods, such as HPLC-PDA and HPLC-UV, require more expensive items and skilled users when compared with the AA-HMDE-DLPME method. In addition to this, the AA-HMDE-DLPME method provides a greener feature than the other methods, as it uses environmentally friendly and inexpensive extracting solvents.

4 . Conclusions

This current research proposed a green and fast air-assisted hydrophobic magnetic deep eutectic solvent dispersive liquid phase microextraction (AA-HMDE-DLPME) method for the extraction and determination of melamine in milk and milk-based products for UV–Vis spectrophotometry analysis. The CCD was used to optimize four extraction factors, including extraction cycles, pH, HMDES-5 volume, and acetone volume, to yield the maximum extraction efficiency for melamine. The method implied the easy preparation of HMDESs in a one-step process without any further purification steps. Interestingly, the HMDES-5 based on octanoic acid, aliquat-336 and CoCl$_2$ was, for the first time, tested as an extracting solvent for the extraction of melamine.

Under optimum extraction conditions, the AA-HMDE-DLPME method presented good analytical features in terms of wide linear range, low LOD, high PF, and good precision/accuracy, and it was successfully applied to the analysis of melamine in milk and milk-based products. In conclusion, the obtained results demonstrate that the developed AA-HMDE-DLPME method was simple to perform, fast, selective, and reliable without requiring organic solvents for the sample preparation procedure for melamine analysis.

CRediT authorship contribution statement

Adil Elik: Supervision, Investigation, Writing - original draft. **Seçkin Fesliyan:** Investigation, Writing – original draft. **Nevcihan Gürsoy:** Conceptualization, Software, Validation. **Hameed Ul Haq:** Conceptualization, Writing – review & editing. **Roberto Castro-Muñoz:** Conceptualization, Writing – review & editing. **Nail Altunay:** Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136573.

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