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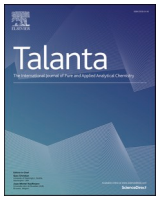

Green infant formula analysis: Optimizing headspace solid-phase microextraction of carbonyl compounds associated with lipid peroxidation using GC-MS and pentafluorophenylhydrazine derivatization

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ABSTRACT

The refinement and optimization of a method combining headspace solid-phase microextraction (HS-SPME) with gas chromatography-mass spectrometry (GC-MS) was successfully performed for the first time to determine seven carbonyl and dicarbonyl compounds, including glyoxal, methylglyoxal, dimethylglyoxal, and malondialdehyde in infant formulae, related to lipid peroxidation. HS-SPME was utilized for simultaneous extraction and derivatization with pentafluorophenylhydrazine (PFPH). Critical parameters such as temperature, pH, extractive phase, and salting-out were meticulously investigated and fine-tuned by an asymmetrical $2^2 3^2 // 9$ screening design to ensure the method's efficacy and reliability. Optimal conditions included a PFPH concentration of 5 g/L, pH 5.0, head-space extraction at 60 °C within 10 min, utilizing a DVB/CAR/PDMS coating, and a 20% w/w salting-out. The analytical validation of this method, compliant with FDA guidelines, demonstrated exceptional linearity, sensitivity, specificity, precision (RSD ≤13.8%), and accuracy (84.8% ≤ recovery ≤111.5%). The metric approach AGREEprep confirms its eco-friendliness, marking a significant step towards an environmentally conscious approach in infant formula analysis. An occurrence study conducted on 25 infant formula samples revealed widespread carbonyl and dicarbonyl compounds in both powdered and liquid variants. ANOVA results exhibited variations in compound concentrations among different sample groups. Clustering analyses delineated distinct groups based on carbonyl content, indicating the potential of these compounds as markers for lipid peroxidation and food quality assessment. This method serves as a valuable tool for evaluating infant formula quality, stability towards oxidation, and safety.

Abbreviations: α-Dicarbonyl Compounds (**α-DDC**), Acceptable Daily Intake (**ADI**); Acetaldehyde (**ACE**), Analysis of Variance (**ANOVA**); Benzaldehyde (**PhCHO**), Carboxen (**CAR**); Dimethylglyoxal (**DMGO**), Divinylbenzene (**DVB**); European Food Safety Authority (**EFSA**), Flame Ionization Detector (**FID**); Food Additives, Flavourings; Processing Aids, and Materials in Contact with Food (**AFC**); Food and Drug Administration (**FDA**), Formaldehyde (**FCHO**); Gas Chromatography (**GC**), Generally Recognized as Safe (**GRAS**); Glyoxal (**GO**), Head-Space (**HS**); High-Performance Liquid Chromatography (**HPLC**), Infant Formula (**IF**); International Agency for Research on Cancer (**IARC**), Joint FAO/WHO Expert Committee on Food Additives (**JECFA**); Limit of Detection (**LOD**), Liquid Follow-up IF (**LFIF**); Liquid Starter IF (**LSIF**), Malondialdehyde (**MDA**); Mass Spectrometry (**MS**), Methylglyoxal (**MGO**); Polyacrylate (**PA**), Polydimethylsiloxane (**PDMS**); Polyunsaturated Fatty Acids (**PUFA**), Powdered Follow-up IF (**PFIF**); Powdered Starter IF (**PSIF**), Quality Control Samples (**QCs**); Reactive Oxygen Species (**ROS**), Relative Standard Deviation (**% RSD**); Solid Phase Microextraction (**SPME**), Lower Limit of Quantification (**LLOQ**); Threshold of Toxicological Concern (**TTC**), Ultra-high temperature processing (**UHT**); Upper Limit of Quantification (**ULOQ**), World Health Organization (**WHO**).

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1. Introduction

Infant formula (IF) plays a pivotal role in ensuring the nutrition and well-being of infants, particularly when breastfeeding is not feasible, according to guidelines set forth by the World Health Organization (WHO) [1]. IF serves as a vital alternative or supplementary source of nutrition during the critical first 12 months of a child's life [1,2]. Ensuring IF safety is crucial, given their exposure to rigorous manufacturing processes, including Ultra-high temperature processing (UHT) at up to 135 °C for 2–5 s to eliminate biological hazards [2]. However, these processes can generate hazardous organic compounds through lipid peroxidation and the Maillard reaction. Lipid peroxidation is a highly aggressive reaction in food, affected by external factors like light, UV radiation, heat, and storage conditions. It results in the breakdown of polyunsaturated fatty acids (PUFA) by reactive oxygen species (ROS) through a radical pathway [3]. Simultaneously, the Maillard reaction, a complex process between amino groups and reducing sugars like lactose, occurs varying with pH but share a common Amadori rearrangement [4]. The primary concern associated with these reactions is not only the reduction in the nutritional value of food products but also the formation of α-dicarbonyl compounds (α-DDC), including glyoxal (GO), methylglyoxal (MGO), and dimethylglyoxal (DMGO), as well as the occurrence of various unsaturated and saturated carbonyl compounds, including malondialdehyde (MDA), formaldehyde (FCHO), acetaldehyde (ACE), and benzaldehyde (PhCHO), raising concerns about their potential health risks since they have been documented to be linked to various inflammatory conditions and are potentially implicated in the development of chronic diseases such as diabetes, kidney disease, and Alzheimer's disease, among others [4,5].

Regulatory organizations such as the International Agency for Research on Cancer (IARC) categorize compounds based on their carcinogenic potential to humans. Formaldehyde is in Category 1, indicating strong evidence of its carcinogenicity. ACE is in Category 2B, suggesting possible carcinogenicity to humans. MDA, GO, and MGO are Unclassifiable in terms of human carcinogenicity (Category 3) due to insufficient toxicological data [6,7]. The European Food Safety Authority (EFSA) has established a Threshold of Toxicological Concern (TTC) for MDA consumption at 30 μg/kg of body weight (bw) per day [8]. Similarly, the WHO has set a tolerable intake of 0.2 mg/kg of bw per day for lifelong oral exposure to GO [9]. The WHO has also defined a TDI at 0.15 mg/kg of bw, confirmed by the Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food (AFC) of the EFSA [10]. ACO, DMGO, and PhCHO are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA), while ACE and PhCHO are accepted as flavoring substances in the European Union [11–13]. Additionally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set an Acceptable Daily Intake (ADI) of 5 and 900 mg/kg of bw per day for PhChO and DMGO, respectively [10,14]. Additionally, lifetime acceptable intake of 185 μg/day has been established for ACE [15].

Given the food safety concerns associated with these reactions, adopting a precise analytical approach is crucial in food analysis. Microextractions offer accurate carbonyl compound analysis with benefits like sample preservation, trace compound sensitivity, eco-friendly solvent use, faster extraction, automation, and selectivity [16]. Solid Phase Microextraction (SPME) is a reliable method for volatile and semi-volatile compound analysis in complex foods, simplifying sample preparation, reducing solvent use, and providing high sensitivity and quantitative analysis [16,17]. Its efficiency, shorter extraction times, and lower contamination risk make it ideal for precise carbonyl compound analysis in complex food [16–18].

SPME also enables in-fiber and in-solution derivatization reactions, commonly used for carbonyl compound analysis [16–18]. Derivatization of carbonyl compounds commonly use hydrazine and hydrazine-derivates due to their reactivity, stable derivatives, increased sensitivity, and compatibility with various analytical techniques, including high-performance liquid chromatography (HPLC) and gas chromatography (GC) with different detection systems [16,19]. SPME has already demonstrated its effectiveness in analyzing other volatile compounds in infant food products. García-Llatas et al., employed SPME for GC-mass spectrometry (MS) analysis of pentanal, hexanal, heptanal and pentane in liquid milk-cereal-based infant foods [20]. Subsequently, SPME was used for analyzing hexanal and pentane in liquid infant foods and powdered formulas by GC-flame ionization detector (FID), focusing on monitoring lipid peroxidation. García-Llatas et al. utilized a carboxen/polydimethylsiloxane SPME fiber for 45-min head-space (HS) extraction in both studies [21].

Here, we present a novel approach for simultaneously determining seven carbonyl compounds in IF. This methodology involves extracting and derivatizing of formaldehyde, malondialdehyde, acetaldehyde, glyoxal, methyl glyoxal, dimethyl glyoxal and benzaldehyde using HS-SPME and GC-MS. This work presents a different SPME approach over the existing ones for analyzing volatile compounds in infant formula and baby food via GC-MS and GC-FID [20,21]. The study explores various SPME fiber coatings and different hydrazines as derivative reagents. Notably, it evaluates in-sample derivatization simultaneously with analyte extraction to enhance sensitivity, deviating from the common pre-extraction derivatization procedure. The HS-SPME-GC-MS method was validated following the guidelines set by the FDA [22,23]. It achieved excellent results in terms of linearity, specificity, accuracy, and precision. The greenness of the method is confirmed by the novel AGREEprep tool. An occurrence study was conducted on 25 infant formula samples, shedding light on their carbonyl compound content. This allowed their segregation into three different classes through the use of Hierarchical Cluster Analysis and Principal Component Analysis. The present methodology can be used to study the degree of lipid peroxidation of infant formulae and the impact of thermal treatment by assessing potential variations in their carbonyl compound content.

2. Materials and methods

2.1. Chemicals and materials

A range of high-purity (≥97%) chemicals and reagents were purchased for this study, including hyper grade acetic acid (AAc, Cas No. 64-19-7), ammonium acetate (AA, Cas No. 631-61-8), ammonium formate (AF, Cas No. 540-69-2), ammonium sulfate (AS, CAS No: 7783-20-2), dinitrophenylhydrazine (DNPH, CAS No. 119-26-6), formic acid (FAc, Cas No. 64-18-6), methanol (MeOH, CAS No: 6-56-1), pentafluorophenylhydrazine (PFPH, CAS No. 828-73-9), and reagent grade, and phenylhydrazine (PH, Cas No. 100-63-0) from Merck (Darmstadt, Germany). Additionally, hydrochloric acid (HCl, CAS no. 7647-07-0) with a purity of 37% (w/w) was purchased from VWR Chemicals (Pennsylvania, USA), while Milli-Q water was generated using a Millipore purification system (Millipore, Billerica, MA, USA). To create standard solutions for analysis, various analytical standards including acetaldehyde (ACE, CAS no. 75-07-0); benzaldehyde (PhCHO, CAS no. 100-52-7); dimethylglyoxal (DMGO, CAS no. 431-03-8); formaldehyde (FCHO, CAS no. 50-00-0); glyoxal (GO, CAS no. 107-22-2); malondialdehyde (MDA, CAS no. 100683-54-3), and methylglyoxal (MGO, CAS no. 78-98-8) were purchased from Merck. These chemicals were dissolved in methanol (MeOH) at specific concentrations and stored at −20 °C. Deuterated acetaldehyde (ACEd4, CAS no. 1632-89-9), and acetone (ACOd6, CAS no. 666-52-4); were also purchased from Merck (Darmstadt, Germany), used as internal standards (IS) at a concentration of 0.5 ng/mL, and stored at −20 °C.

SPME fibers with different coating materials were acquired from Merck, including fibers with coatings of 100 μm polydimethyl siloxane (PDMS), 85 μm polyacrylate (PA), 75 μm Carboxen/PDMS (CAR/PDMS), 50/30 μm divinylbenzene/CAR/PDMS (DVB/CAR/PDMS), and 65 μm PDMS/DVB. To ensure their optimal performance, all fibers underwent a conditioning process as per the manufacturer's instructions

before their initial use. Manual sampling was carried out using a manual holder, also procured from Merck. This study employed different laboratory equipment, including a Centromix II-BL Centrifuge from J. P. Selecta (Barcelona, Spain), a pH meter model Basic 20 from Crison Instruments (Barcelona, Spain) a 2510EMTH ultrasonic bath from Branson Ultrasonics (Danbury, USA), and a Reax top vortex mixer from Instruments GmbH & Co (Schwalbach, Germany) were used to develop all experiments.

2.2. Samples

A total of 25 infant formula (IF) samples were acquired for this study, comprising powdered starter IF (PSIF, n = 7), powdered follow-up IF (PFIF, n = 7), liquid starter IF (LSIF, n = 4), and liquid follow-up IF (LFIF, n = 7). These samples were purchased from parapharmacies and local supermarkets in Santiago de Compostela, Spain. All samples were stored in their original packaging until the study. Liquid samples were stored at 4 °C and opened just before analysis, while powder samples were safeguarded from light and reconstituted at a concentration of 15% w/v using boiled drinking water to form the sample solution, adhering to the manufacturer's guidelines. Additionally, quality control samples (QCs) were freshly prepared by adding standard solutions to an initial infant formula sample at 250, 500, and 1000 ng/mL. These QCs were then stored at −20 °C for no more than one week, preserving their integrity and consistency for subsequent analytical assessments.

2.3. Head-space solid-phase microextraction

Simultaneous extraction of ACE, DMGO, FCHO, GO, MDA, MGO, and PhCHO from infant formulae was accomplished using HS-SPME. In a concise description of the procedure, 2.5 mL of the sample solution (or liquid infant formula), which included the internal standards, was placed into a conical-bottom centrifuge tube. Subsequently, 20% w/v of ammonium sulfate was added, and the mixture was vortexed for 1 min and then centrifuged at 3500 rpm for 5 min. Following this, a 0.5 mL aliquot of the aqueous phase was transferred to a 6 mL head-space vial. In this vial, 0.1 mL of PFPH (5 g/L in 215 mmol/L HCl) solution was added, and the volume was adjusted to 2.15 mL with MilliQ water obtaining a pH 5.0. A magnetic bar was placed inside the vial, which was then sealed with an aluminum cap and septum. The system was vortexed at room temperature for 1 min. The SPME holder's needle pierced the septum, and the vial was placed in a water bath set at 60 °C. The 50/30 μm CAR/PDMS/DVB fiber was exposed to the headspace for 10 min. The analytes were desorbed at the GC-MS inlet, which was maintained at 270 °C, for 5 min. A schematic representation of the HS-SPME process is depicted in Fig. 1.

2.4. GC-MS analysis

GC-MS analysis was carried out employing an Agilent Technologies 7890B–5977B-MSD system based in California, USA. For the inlet, a straight ultra-inert liner (Model 5190-4048) with a 0.755 mm inner diameter was used in splitless mode, with the inlet temperature maintained at 270 °C and a He flow rate of 1 mL/min. The analyte separation was achieved using an Agilent Technologies 2A J&W HP-5MS column, measuring 30 m in length, 0.25 mm in inner diameter, and with a film thickness of 0.25 μm. The oven temperature program initially started at 80 °C and was held for 1 min, then ramped up at a rate of 3 °C per minute to reach 150 °C, and finally increased to 280 °C with a rate of 30 °C per minute, where it was held for 3 min.

For analyte detection, a single quadrupole mass analyzer was utilized, operating in the positive ion mode (EI+) with an electron impact source set at 70 eV. The temperatures for the transfer line, source, and quadrupole were set at 280 °C, 230 °C, and 150 °C, respectively. Data acquisition and analysis were carried out using MassHunter Workstation Software, version B.07.00, provided by Agilent Technologies in California, USA. The total analysis time for this procedure was 15.33 min.

2.5. Statistical analysis

An asymmetric $2^2 3^2//9$ screening design was employed to investigate the influential factors of the methodology in a total of 9 experiments [24]. This design choice significantly reduced the number of required runs compared to a full factorial design, which would have necessitated of 36 experiments. The factors under scrutiny encompassed extraction/derivatization temperature (40 and 60 °C), SPME coating type (PDMS/DVB and DVB/CAR/PDMS), pH levels (3.0, 5.0, and 7.0), and the concentration of the salting-out agent (0.17, and 20% w/v).

This design characterizes how substituting one level of a particular factor for another affects the SPME method, with response as the obtained peak area in the GC-MS analysis. The effects were represented graphically in the form of total effects and delta weigh plots. The variables indicate the presence or absence of specific factors. For instance, the coefficient $b_{1/21}$ defines the impact of replacing an extraction temperature of 40 °C with 60 °C, and similarly, $b_{2/21}$ describes the effect of replacing PDMS/DVB coating with DVB/CAR/PDMS, and so forth. To

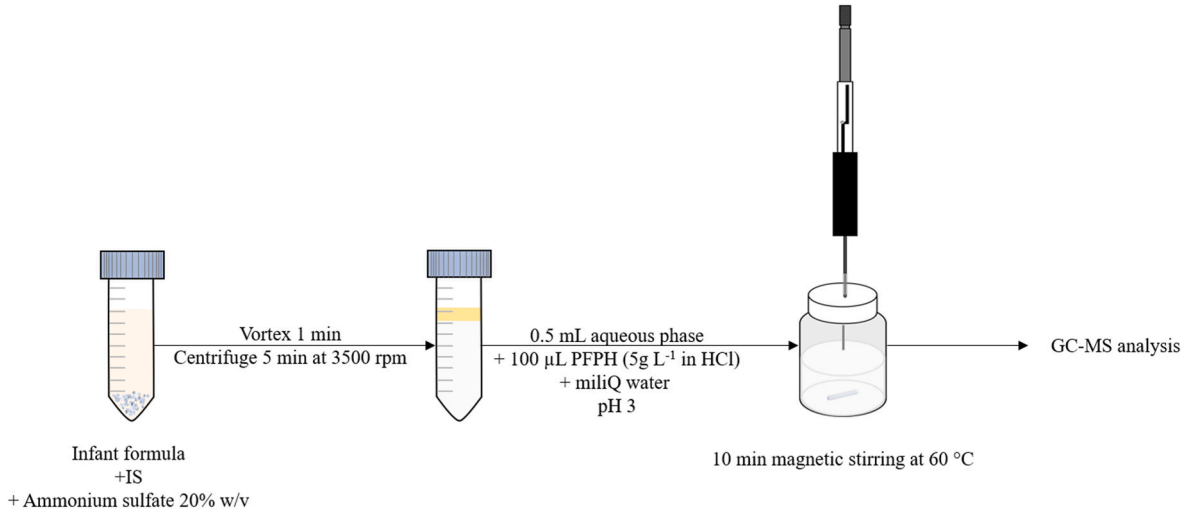

Fig. 1. Scheme of the head-space solid-phase microextraction of carbonyl and α-dicarbonyl compounds for simultaneous GC-MS determination in infant formulae. IS, internal standard; PFPH, pentafluorophenylhydrazine.

generate the experimental design, assess the experimental data, and visualize its effects, NemrodW® statistical software (version 2011) was utilized.

The experimental data related to the presence of carbonyl compounds in infant formula underwent a comprehensive statistical analysis using IBM SPSS Statistics software. This analysis aimed to characterize the data and uncover insights into the differences and groupings within the dataset. Analysis of Variance (ANOVA) was utilized to determine whether significant variations existed among different types or brands of infant formula. A Box and Whisker Plot was employed to summarize the spread of count-based datasets by displaying quartiles, the median, and any outliers. The box represents the interquartile range, while whiskers show data within 1.5 times the interquartile range, with outliers being marked. K-means clustering was used to identify natural groupings in the occurrence data, dividing it into clusters based on similarities. Additionally, hierarchical cluster analysis (HCA) created a hierarchical structure of clusters to reveal relationships between carbonyl compound profiles in different infant formulae.

2.6. Analytical performance of the method

A PSIF sample served as the blank specimen for conducting analytical validation of the method, following the guidelines outlined by the Food and Drug Administration (FDA) for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products [22], as well as the Bioanalytical Method Validation Guidance for Industry [23].

To establish the method's specificity, a quantifier ion and two qualifier ions were chosen for all analytes and internal standards, each eluting at different retention times. The absence of any interference in these elution regions confirmed the method's specificity. Individual standard addition was performed alongside internal standard calibration curve construction, ranging from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ) for each analyte. These curves were utilized to determine the limit of detection (LOD) and LLOQ according to eq. (2).

$$Y_{LOD_{/LLOQ}} = \overline{Y}_{blank} + {}^{3.3}\!/_{10}\sigma_{blank} \quad \text{(eq 2)}$$

Additionally, LLOQ and ULOQ represent the lowest and highest concentrations of each analyte that can be quantitatively determined with acceptable precision and accuracy. Therefore, calibration curves were established at six concentration levels (n = 3) and used to evaluate the linearity of the method. Furthermore, the sensitivity at LLOQ was assessed as the calibration slope divided by the relative standard deviation (%RSD) for each analyte in triplicate. QCs (refer to *Samples* section) were employed to evaluate the accuracy of the method, involving recovery (n = 3), and precision through intraday and interday assays, measured in terms of %RSD (n = 5).

Matrix effect was assessed through a comparison of slope values of the calibration curve with those obtained from the standard addition calibration curve in in the selected group of samples. Moreover, to assure the applicability of the developed method to the different group of samples a matrix extension study was performed for power follow-up, liquid starter and liquid follow-up formulae at three levels 250, 500 and 100 ng/mL in quintuplicated.

3. Results and discussion

3.1. Parameters optimization of head-space solid-phase microextraction methodology

The choice of a suitable derivatization reagent is crucial for the method's effectiveness. It's essential that this reagent forms a stable product when reacting with the analyte and is compatible with the chosen separation and detection techniques. The reaction between the reagent and analyte should also occur rapidly, which is particularly important in GC analysis due to the need for undecomposed evaporation of derivatives. Common derivatization reactions for carbonyl compounds in GC involve forming oximes and hydrazones [27,32,35,36]. In our study, we evaluated three hydrazine reagents (DNPH, PFPH, and PH) for their ability to create hydrazones with specific carbonyl compounds (MDA, GO, MGO, DMGO, and ACOd6) in triplicate. As shown in Fig. 1SA (Supplementary material), the highest signal, in terms of relative chromatographic area, was achieved when PFPH was used as the derivative reagent. Consequently, PFPH was chosen for further analysis. The key difference between PFPH, DNPH, and PH lies in the nature of the substituents on the phenyl ring (Fig. 2S). PFPH contains fluorine atoms on the phenyl ring, which can enhance stability and reactivity. The increased polarity of these derivatives can lead to improved resolution in chromatograms [35]. In contrast, DNPH, characterized by a phenyl ring with nitro groups, is recognized for its reaction with carbonyl compounds, yielding colored derivatives utilized in UV/vis or fluorescence spectroscopy applications following liquid chromatographic separation; nevertheless, recent innovations have introduced gas chromatographic quantification methods for DNPH derivatives [25,26].

The concentration of derivative reagent was then studied at 1, 3 and 5 g/L based on literature (Fig. 1S.B). For this application the highest signal in terms of relative chromatographic area was achieved using 5 g/L; therefore, this concentration of PFPH was selected. Hydrazine derivatization reactions require acidic conditions [25,26,35,38], and different acids were tested to achieve a pH of 5.0 during the derivatization procedure. In Fig. 1S.C, it is evident that the highest relative chromatographic areas are obtained when HCl is used. This difference arises from the fact that strong acids almost completely donate their protons to water molecules, whereas weak acids establish an equilibrium between undissociated acid molecules and ions in solution. This behavior is quantified by the acid dissociation constant (K_a). Therefore, strong acids exhibit a higher degree of ionization compared to weak acids at the same pH, which affects their reactivity and behavior in chemical reactions. Different ammonium salts—ammonium sulfate (AS), ammonium formate (AF), and ammonium acetate (AA)—were rigorously tested in triplicate as salting-out agents (Fig. 3SA). The results indicated that AS yielded the most promising outcomes in terms of relative chromatographic area, with AS consistently surpassing 80% relative areas across most analytes. Notably, while MGO showed higher areas with AA, AS still achieved relative areas exceeding 80%. Thus, AS was chosen as the primary salting-out agent for subsequent research endeavors. Furthermore, the impact of agitation methods—ultrasound versus magnetic stirring—was investigated (Fig. 3B). The findings revealed that magnetic stirring consistently resulted in superior relative chromatographic areas for all analytes. As a result, the decision was made to conduct further experiments utilizing magnetic stirring as the preferred agitation method.

We conducted tests using various SPME fibers, each coated with different materials, following PFPH derivatization under the conditions outlined above. This derivatization was carried out for 15 min in the headspace of the vials. The coatings tested included PDMS, PA, CAR/PDMS, DVB/CAR/PDMS, and PDMS/DVB. These tests aimed to assess their performance in extracting the MDA-PFPH, GO-PFPH, MGO-PFPH and DMGO-PFPH derivatives in triplicate (Fig. 4S). DVB/CAR/PDMS and PDMS/DVB coatings demonstrated the highest relative areas, showcasing their effectiveness in retaining these compounds. Additionally, we investigated the kinetics of simultaneous derivatization and extraction using the DVB/CAR/PDMS fiber (Fig. 5S). It was observed that equilibrium is achieved after 10 min of the reaction for these analytes under the given conditions. Therefore, these conditions were selected for further experiments to examine the key parameters influencing the performance of HS-SPME during the simultaneous extraction and PFPH derivatization of FCHO, MDA, ACE, ACEd4, ACOd6, GO, MGO, DMGO, and PhCHO. This evaluation was carried out using an asymmetric screening design, considering the following factors: b_1,

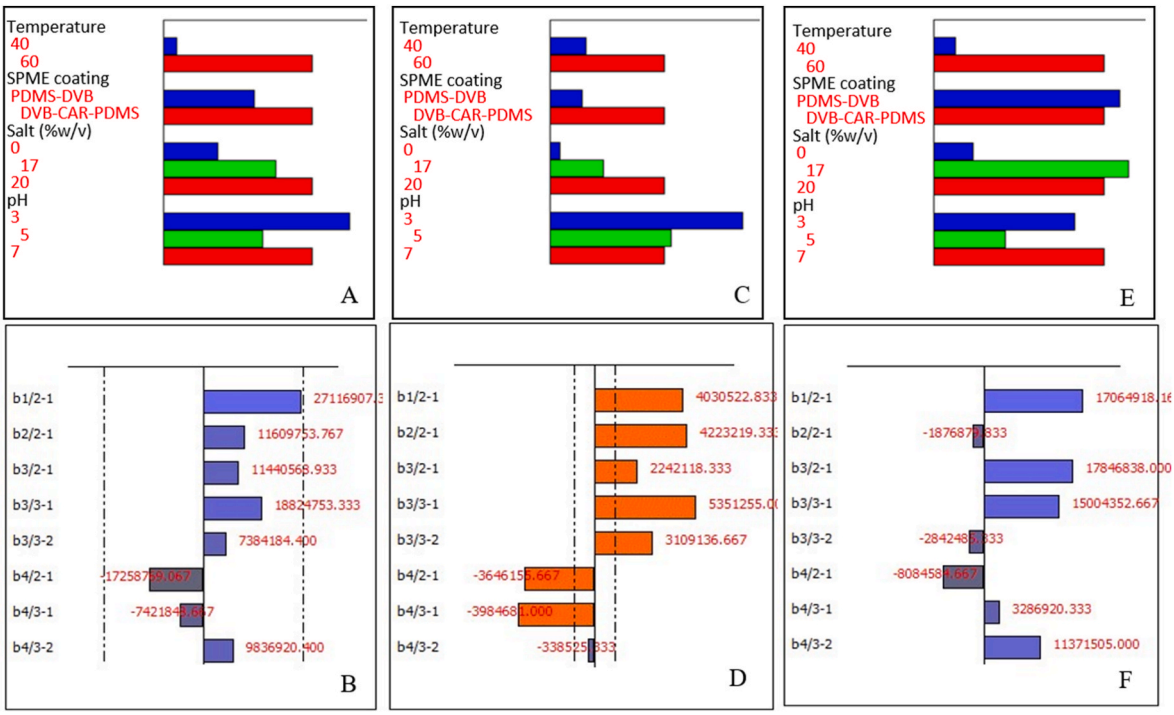

Fig. 2. Total effects and delta weigh plots of the asymmetrical 2232//9 screening design for malondialdehyde (A, B), deuterated acetone (C, D), glyoxal (E, F).

temperature; b_2, SPME coating material; b_3, concentration of the salting-out agent; and b_4, pH. To minimize random errors, all experiments were conducted randomly. The results were analyzed using total effect and delta weight plots [24], which depict the differential effects of each factor when considering pairwise combinations of two different levels. In delta plots, the length of the bars reflects the relative magnitude of the effects. Positive values indicate effects favoring higher levels, while negative values indicate effects favoring lower levels. Dotted lines on the plots represent the statistical significance levels, determined using the Length's method at a 95% confidence level. An effect is considered statistically significant when it surpasses this reference line. By considering the sign and value of the effects, we were able to select the optimal level for each experimental parameter. In total effect plots, the length of the bars directly correlates with the effect of each factor on the analytical response. Fig. 2 illustrates the findings from the delta plots revealing that none of the parameters were statistically significant for malondialdehyde (Fig. 2B), glyoxal (Fig. 2F) and dicarbonyl compounds All of variables exhibited statistical significance for aldehydes as deuterated acetone (Fig. 2D). Remarkably, all analytes displayed similar behavior regarding these factors. Despite these varied responses, the total effect chart (Fig. 2A, **C, E**) indicated that the best results were achieved when conducting the HS-SPME at 60 °C, utilizing a DVB/CAR/PDMS coating on the SPME fiber, and employing a 20% w/w concentration of the salting-out agent. According to the pareto charts, a pH of 3.0 was optimal for aldehydes (Fig. 2A–C), and for dicarbonyl compounds, pH 7.0 yielded the highest response, followed by pH 3.0 (Fig. 2E). Since the selection of pH was statistically significant for aldehydes (Fig. 2D), it was decided to utilize a sample solution with a pH of 3.0.

3.2. Analytical validation of the HS-SPME-GC-MS methodology

Following FDA guidelines [22,23], we conducted a comprehensive evaluation of the HS-SPME procedure's analytical features. Initially, we assessed the selectivity and specificity by selecting one quantifier and two qualifier ions with specific retention times for FCHO, MDA, ACE, ACEd4, ACOd6, GO, MGO, DMGO, and PhCHO. It is worthwhile pointing out that all the target analytes and IS exhibited distinctive retention times, and no matrix interferences were observed in the GC-MS chromatogram (Fig. 3).

The method's characteristics, including determination limits, linearity, and sensitivity, are summarized in Table 1. ACEd4 acted as the IS for FCHO, MDA, ACE, and PhCHO, while ACOd6 was employed for GO, MGO, and DMGO. By employing standard addition with IS calibration plots for each analyte, we covered a concentration range from 50 to 1500 ng/mL, with the IS concentration fixed at 500 ng/mL. This approach showcased remarkable linearity ($r^2 \geq 0.9990$). Furthermore, when assessing sensitivity at the lower limit of quantification (LLOQ), we achieved a relative standard deviation (RSD) of ≤7.6%. We used QC samples to evaluate the method's accuracy and precision, as outlined in Table 2. Our accuracy results fell within the range of 84.8%–111.5% of recoveries, and both intraday and interday precision exhibited excellent outcomes, with RSD values of ≤13.8% and ≤12.1%, respectively, meeting the established criteria for acceptability.

Table 3 summarizes the results obtained from the assessment of the Matrix Effect and Matrix Extension Study. Notably, a significant matrix effect is evident for all the analytes when evaluated in powdered starter infant formulae, as compared to follow-up powdered infant formula and liquid infant formulae. This observed matrix effect aligns with prior findings in the context of SPME and is likely attributed to the presence of interfering compounds which may compete with the target analytes for binding sites on the SPME fiber or influence the chemical equilibrium during the extraction process, as reported in previous studies [16–18,20,21].

Given that such interference can introduce variations in extraction efficiency and potentially lead to inaccurate quantification of the analytes, it becomes imperative to employ specific calibration curves for each distinct group of samples within the set [23]. On the other hand, the Matrix Extension Study, as presented in Table 3, demonstrates acceptable accuracy (80–116% recovery) and precision (0.4–19.0 % RSD) across the different sample groups. This outcome underscores the applicability of the HS-SPME-GC-MS method to the entire set of samples [23].

Comparatively, previously published articles (see Table 1S in the supplementary material) on chromatographic methods for carbonyl

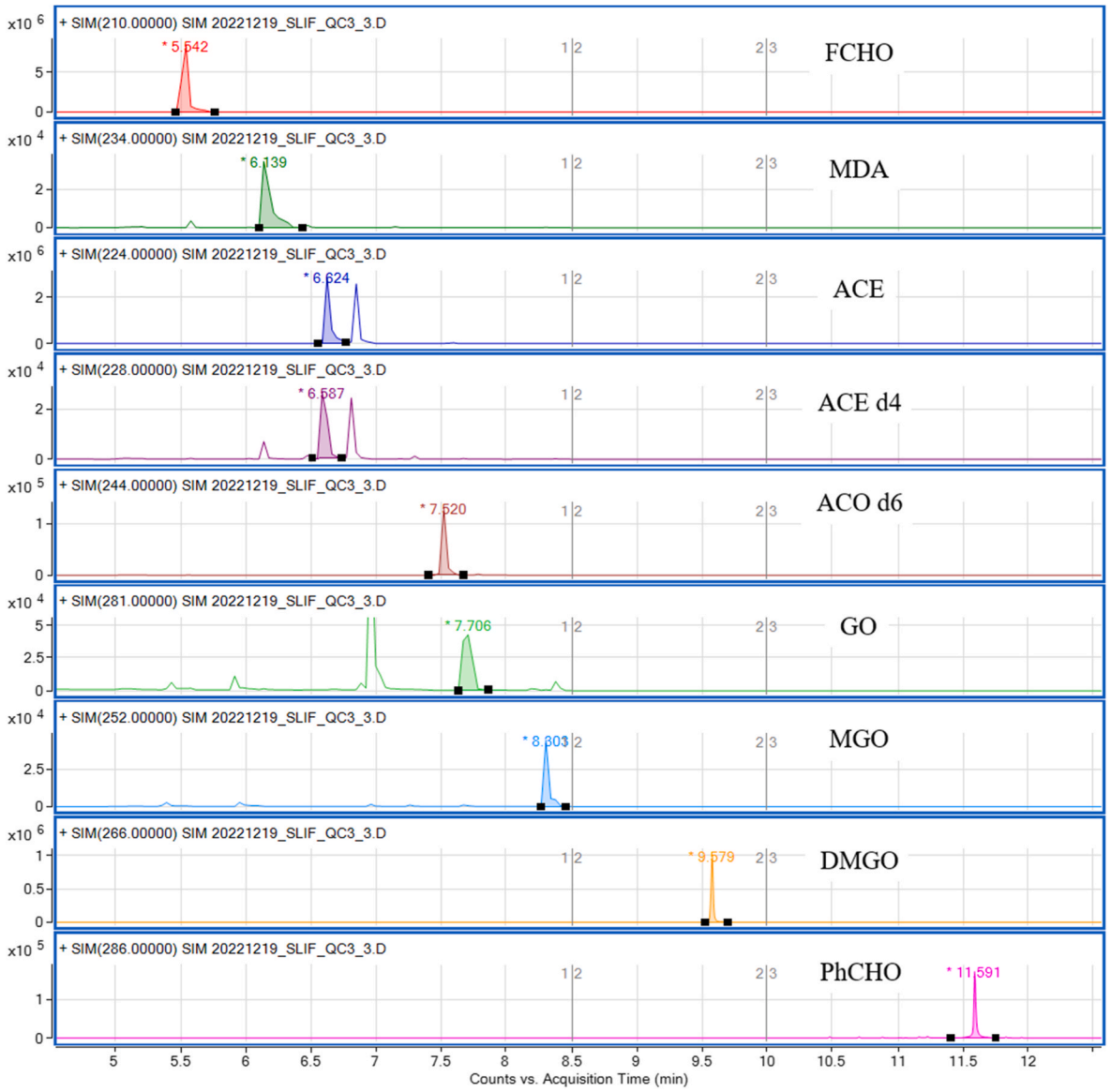

Fig. 3. Chromatogram of formaldehyde (FCHO), malondialdehyde (MDA), acetaldehyde (ACE), deuterated acetaldehyde (ACEd4), deuterated acetone (ACOd6), glyoxal (GO), methylglyoxal (MGO), dimethylglyoxal (DMGO), benzaldehyde (PhCHO) at 1μ/mL of powder starter infant formula.

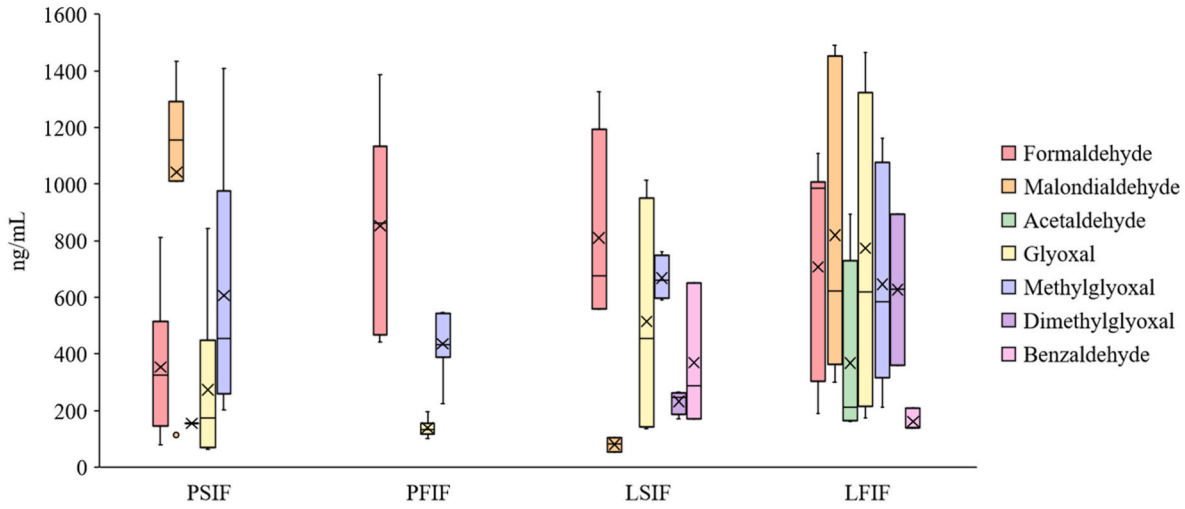

Fig. 4. A box and whisker plot of the variability in carbonyl compounds concentrations among positive samples.

Table 1
Specificity, sensibility, linearity and determination limits of the HS-SPME method for simultaneous GC-MS determination of carbonyl and α-dicarbonyl compounds in infant formulae.

Analyte	RT	Quantifier ion	Qualifier ions		R^2	LOD	LLOQ	ULOQ	Sensitivity
	min	*m/z*	*m/z*	*m/z*		ng/mL	ng/mL	ng/mL	% RSD
FCHO	4.65	210	211	182	0.9990	16	50	1500	6.5
MDA	5.32	234	233	215	0.9999	20	50	1500	7.6
ACE	5.77	224	182	196	0.9993	50	150	1500	6.9
ACEd4[a]	5.73	228	227	229	–	–	–	–	–
ACOd6[a]	6.55	244	242	245	–	–	–	–	–
GO	6.55	281	182	155	0.9995	15	50	1500	2.1
MGO	7.74	252	253	182	0.9992	15	50	1500	2.5
DMGO	8.97	266	267	223	0.9996	16	50	1500	2.1
PhCHO	11.15	286	287	285	0.9998	50	150	1500	2.7

[a] Used as internal standard; RT, retention time; LOD, limit of detection; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; RSD, relative standard deviation; FCHO, formaldehyde; MDA, malondialdehyde; ACE, acetaldehyde; ACEd4, deuterated acetaldehyde; ACOd6, deuterated acetone; GO, glyoxal; MGO, methylglyoxal; DMGO, dimethylglyoxal; PhCHO, benzaldehyde.

Table 2
Accuracy and Precision of the HS-SPME Method for Simultaneous GC-MS Determination of Carbonyl and α-Dicarbonyl Compounds in Infant Formulae.

Analyte	Accuracy			Precision					
	(n = 3) % Recovery			Intraday (n = 5) % RSD			Interday (n = 5) % RSD		
	250 ng/mL	500 ng/mL	1000 ng/mL	250 ng/mL	500 ng/mL	1000 ng/mL	250 ng/mL	500 ng/mL	1000 ng/mL
FCHO	86.8	93.1	97.9	14.7	7.7	12.8	9.6	5.2	3.2
MDA	105.8	110.8	110.1	13.7	6.6	13.8	8.3	9.1	12.1
ACE	94.4	100.5	98.5	13.1	11.5	4.3	5.7	4.9	4.6
GO	89.5	101.3	98.6	7.8	8.2	9.6	5.2	3.7	4.5
MGO	84.8	104.5	105.5	13.9	7.2	9.0	4.1	5.2	12.1
DMGO	108.2	111.3	101.1	13.4	9.4	4.9	6.6	11.1	14.1
PhCHO	105.1	102.6	111.5	9.6	7.6	11.9	5.1	3.5	11.8

RSD, relative standard deviation; FCHO, formaldehyde; MDA, malondialdehyde; ACE, acetaldehyde; GO, glyoxal; MGO, methylglyoxal; DMGO, dimethylglyoxal; PhCHO, benzaldehyde.

Table 3
Infant Formulae's matrix effect and matrix extension study.

Analyte	Power follow-up IF			Liquid starter IF			Liquid follow-up IF		
	Matix effect[a]	Accuracy[b]	Precision[c]	Matix effect[a]	Accuracy[b]	Precision[c]	Matix effect[a]	Accuracy[b]	Precision[c]
	%	% Recovery	% RSD	%	% Recovery	% RSD	%	% Recovery	% RSD
FCHO	43.7	85–104	0.7–4.0	25.5	80–103	0.7–1.1	49.8	83–113	1.5–8.7
MDA	123.7	80–112	4.6–11.2	21.6	83–107	1.7–5.0	9.3	90–107	1.7–3.8
ACE	57.2	91–102	2.0–4.3	48.7	92–104	3.8–6.4	26.1	85–111	3.9–6.7
GO	19.6	102–113	4.7–12.7	21.5	88–106	2.3–9.2	14.8	89–108	1.8–3.8
MGO	1.63	92–115	0.7–5.1	7.13	88–103	0.5–1.5	3.8	98–118	0.4–6.9
DMGO	6.69	84–104	2.1–19.0	1.57	92–109	0.8–3.7	3.9	90–104	1.2–12.4
PhCHO	149	91–116	2.7–5.4	15.1	96–108	0.5–5.2	20.9	91–111	4.4–5.2

IF, infant formula; RSD, relative standard deviation; FCHO, formaldehyde; MDA, malondialdehyde; ACE, acetaldehyde; GO, glyoxal; MGO, methylglyoxal; DMGO, dimethylglyoxal; PhCHO, benzaldehyde; [a] determined through a comparison of slope values with those obtained from the standard addition calibration curve in powder starter infant formula; [b] assessed at three different concentration levels (250, 500 and 100 ng/mL) in triplicate; [c] assessed in intra- and inter-day assays at three levels of concentration (250, 500 and 100 ng/mL) in quintuplicate.

compound analysis reported quantification limits either similar to or higher than those presented here [7,25–32]. Notably, Paiano et al. [33] achieved lower instrumental determination limits than our study in the single analysis of ACE in alcoholic and non-alcoholic beverages using the HS-SPME-GCMS method previously developed by Wang et al. [34], which also exhibited similar linearity but higher variation coefficients. Similarly, Wang et al. [35] reported lower limits in the single GC-MS analysis of MDA in meat using hydrolysis and PFPH derivatization, with recoveries and precision on par with our findings. Finally, Lim & Shim [36] reported lower determination limits than our study in the simultaneous GC-MS analysis of GO and MGO in alcoholic beverages via HS-SPME. Parallelly, the utilization of pentafluoro benzyl hydroxylamine derivatization has demonstrated improvement in the extraction of carbonyl compounds using SPME from alcoholic beverages. Yu et al. [37] reported lower quantification limits for GO, MGO, DMGO, and PhCHO in their targeted metabolomics analyses of Huangjiu, a traditional Chinese alcoholic beverage. Piergiovanni et al. [38] reported lower limits in the analysis of PhCHO in wines. Moreira et al. [39] also documented lower quantification limits for GO, MGO, DMGO, and PhCHO in port wines. Additionally, Hernandes et al. [40] reported lower limits for ACE and FCHO in craft beer.

Our HS-SPME-GC-MS procedure distinguishes itself by achieving lower detection limits, higher accuracy (approaching 100% recovery), and greater precision (with an RSD of ≤14.7%) when compared to prior investigations involving the determination of MDA and α-dicarbonyl compounds in infant formula and baby food [28,29,41–43]. It is noteworthy that our approach demonstrates the capability to identify and quantify up to seven carbonyl and dicarbonyl compounds, showcasing

superior analytical performance in comparison to previously published methods (Table 1S).

3.3. Analytical greenness metric for HS-SPME-GC-MS

To assess the environmental impact of our sample preparation method, we utilized the AGREEprep assessment through the AGREEprep open access software [44]. This tool helps pinpoint the strengths and weaknesses of methods, thereby facilitating the development of more eco-friendly sample preparation procedures. The AGREEprep assessment involves ten steps, each assigning scores between 0 and 1, where 0 represents the poorest performance and 1 the best. These scores, with default weightings for each criterion, are combined to generate an overall score, also ranging from 0 to 1, where 1 signifies optimal performance.

The environmental impact comparison between sample preparation methods for carbonyl and dicarbonyl compound analysis in infant formula and baby food is outlined in Table 2S (supplementary material). Our developed HS-SPME-GC-MS procedure obtained an overall score of 0.62. This score mirrors its performance, influenced by key factors such as reduced hazardous material volume (score: 0.67), significant material reusability (score: 0.75), the capability to process 6 samples simultaneously within an hour, and semiautomatic system integration (score: 0.50). These collective findings underscore that the presented method provides a more environmentally friendly alternative for extracting carbonyl and dicarbonyl compounds from infant formulae and baby food compared to existing methods [28,29,40].

3.4. Occurrence of carbonyl and dicarbonyl compounds in infant formulae

The innovative HS-SPME-GC-MS method was utilized to detect seven carbonyl and dicarbonyl compounds in various IF samples, including both powdered and liquid starter and follow-up IF. The findings are detailed in Table 4.

Fig. 4 plots the variation in carbonyl compound concentrations among the positive samples. All samples tested positive for MGO, with concentrations ranging from 203.3 to 1409.6 ng/mL. Notably, the lowest and highest MGO concentrations were found in PSIF samples. A significant 96% of the samples also tested positive for FCHO and GO. The highest FCHO concentration was observed in PFIF at 1385.7 ng/mL, while the lowest was in PSIF at 78.9 ng/mL. For GO, the highest concentration was in LFIF at 1464.4 ng/mL, and the lowest in PSIF at 61.4 ng/mL.

Among the samples, 68% tested positive for MDA, with the highest concentration at 1491.5 ng/mL found in LFIF and the lowest at 54.1 ng/mL in LSIF. Remarkably, MDA levels in PFIF samples were non-quantifiable. DMGO and PhCHO were found at quantifiable levels only in the liquid samples, representing 24% of the samples. The highest DMGO concentration, 894.9 ng/mL, was found in LFIF, while the lowest, 171.2 ng/mL, was in LSIF. The highest PhCHO concentration was observed in LSIF at 651.4 ng/mL, and the lowest in LFIF at 137.5 ng/mL. ACE was detected in only 20% of the samples, with the lowest concentration, 155.1 ng/mL, found in one PSIF sample, and the highest, 893.0 ng/mL, in LFIF. Similar results were reported by Pozzo et al. [42] and Cesa [41] regarding MDA content in IF. Wang et al. [43] also reported similar GO content in IF. Similarly, Akıllıoğlu et al. [29] and Kocadağlı et al. [28] reported comparable MGO results in the analysis of IF.

Furthermore, an ANOVA analysis (Table 3S) revealed p-values below 0.05 for the occurrence of FCHO, MDA, GO, and PhCHO, indicating a statistically significant difference among sample groups based on the concentration of these analytes. However, no statistically significant differences were found for ACE, MGO, and DMGO. Fig. 6S presents a dendrogram of similarities of carbonyl content among IF samples using the ward-linkage method. This HCA classified infant formulas into three distinct categories based on the dissimilarities of their carbonyl content. Class 1 comprised all samples of LSIF and PS1F 2, Class 2 included PSIF 1, and PSIF samples 3 to 7, while Class 3 encompassed all follow-up formulas. Similarly, the bar graph of clustering of IF in Fig. 7S provides information about the same three clusters formed using the K-means clustering method. In cluster 1, the infant formulas exhibited high positive values, particularly for acetaldehyde, glyoxal, and dimethylglyoxal. Cluster 2 samples scored positively for FCHO, ACE, MDA, GO, and MGO, but showed negative values for DMGO and PhCHO. On the

Table 4
Carbonyl compound occurrence in infant formulae.

IF	Formaldehyde		Malondialdehyde		Acetaldehyde		Glyoxal		Methylglyoxal		Dimethylglyoxal		Benzaldehyde		C
	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD	ng/mL	±SD	
PSIF 1	413.4	86.0	1432.8	23.1	ND	–	70.2	49.3	499.1	61.2	ND	–	ND	–	2
PSIF 2	812.4	38.1	1030.8	36.0	155.1	6.6	841.8	56.4	1409.6	93.6	ND	–	ND	–	1
PSIF 3	318.8	39.3	1290.5	29.4	ND	–	316.6	24.8	976.2	83.5	ND	–	ND	–	2
PSIF 4	165.8	28.4	1011.4	64.1	ND	–	ND	–	455.6	59.9	ND	–	ND	–	2
PSIF 5	78.9	15.6	1258.3	14.7	ND	–	61.4	32.1	257.9	73.7	ND	–	ND	–	2
PSIF 6	329.6	26.5	1156.1	28.2	ND	–	149.2	88.0	435.1	60.3	ND	–	ND	–	2
PSIF 7	ND	–	113.8	88.3	ND	–	196.6	51.0	203.3	49.0	ND	–	ND	–	2
PFIF 1	468.4	27.1	ND	–	ND	–	196.0	16.91	223.9	33.1	ND	–	ND	–	3
PFIF 2	1132.2	47.8	ND	–	ND	–	118.0	69.89	431.2	25.3	ND	–	ND	–	3
PFIF 3	1115.8	17.5	ND	–	ND	–	131.0	34.73	484.7	13.9	ND	–	ND	–	3
PFIF 4	441.3	28.7	ND	–	ND	–	144.0	76.39	546.7	72.5	ND	–	ND	–	3
PFIF 5	1385.7	42.9	ND	–	ND	–	100.0	75.61	387.5	42.2	ND	–	ND	–	3
PFIF 6	562.2	38.2	ND	–	ND	–	154.0	43.4	432.5	10.5	ND	–	ND	–	3
PFIF 7	861.7	36.8	ND	–	ND	–	125.0	18.84	543.3	61.5	ND	–	ND	–	3
LSIF 1	559.2	21.2	ND	–	ND	–	152.9	42.1	614.6	39.8	257.2	85.3	ND	–	1
LSIF 2	558.6	11.4	82.5	12.7	ND	–	136.8	43.3	589.0	93.6	236.3	80.5	651.4	51.3	1
LSIF 3	1327.6	77.4	54.1	51.6	ND	–	1014.9	31.2	761.3	76.7	264.6	75.5	169.4	54.4	1
LSIF 4	791.3	32.9	104.3	96.5	ND	–	754.5	75.2	706.3	52.7	171.2	19.2	287.5	37.5	1
LFIF 1	187.6	12.7	298.2	47.7	ND	–	436.5	21.8	210.3	21.2	ND	–	ND	–	3
LFIF 2	1001.3	62.7	571.1	78.4	ND	–	1182.1	24.6	1162.1	92.5	ND	–	ND	–	3
LFIF 3	1006.3	57.5	621.5	83.7	893.0	34.6	619.4	29.9	1076.9	54.8	ND	–	ND	–	3
LFIF 4	303.6	31.1	362.0	22.9	ND	–	213.5	38.9	315.0	85.5	ND	–	206.9	95.5	3
LFIF 5	360.9	47.4	933.5	47.4	160.5	19.4	1323.2	42.3	398.8	55.5	894.9	53.8	139.7	65.5	3
LFIF 6	1107.5	19.6	1453.1	17.0	176.9	40.9	1464.4	77.4	769.7	27.1	358.2	57.8	137.5	17.1	3
LFIF 7	986.5	44.5	1491.5	46.2	242.6	65.1	174.6	15.8	584.1	19.1	ND	–	ND	–	3

IF, infant formula sample; C, cluster; PSIF, powdered starter IF; PFIF, powdered follow-up IF; LSIF, liquid starter IF; LFIF, liquid follow-up IF; ND, non-determined (concentration below LLOQ).

other hand, cluster 3 samples displayed negative values for FCHO, ACE, MDA, GO, MGO, and DMGO, with slightly positive values for PhCHO.

This analysis, categorizing different infant formulas based on their carbonyl content, strongly supports the use of carbonyl compounds as markers for lipid peroxidation and food quality. The clusters highlight how carbonyl content variations serve as quality indicators, with lower levels indicating freshness and product quality, and higher levels suggesting possible deterioration or storage issues.

4. Conclusions

The development and optimization of the headspace solid-phase microextraction (HS-SPME) methodology for analyzing carbonyl and dicarbonyl compounds in infant formulae has been a meticulous and fruitful process. Several key parameters were investigated and optimized to ensure the method's effectiveness and reliability.

First and foremost, selecting the right derivatization reagent was crucial, and PFPH proved highly effective, enhancing stability, reactivity, and chromatographic resolution. The optimized PFPH concentration of 5 g/L, along with the use of hydrochloric acid for pH control, ensured optimal conditions. DVB/CAR/PDMS and PDMS/DVB coatings on SPME fibers excelled in extracting PFPH derivatives, reaching equilibrium in 10 min. An asymmetric screening design identified HS-SPME at 60 °C, DVB/CAR/PDMS coating, and a 20% w/w salting-out agent as optimal for simultaneous extraction and derivatization of FCHO, MDA, ACE, GO, MGO, DMGO, and PhCHO. pH variations were considered for specific analytes to optimize method performance.

The analytical validation of the HS-SPME-GC-MS methodology followed FDA guidelines, demonstrating excellent selectivity, specificity, linearity, and sensitivity. The determination limits and precision met established criteria, with recoveries ranging from 84.8% to 111.5%. Importantly, the method's performance surpassed or equaled that of previously published methods for carbonyl compound analysis, making it a robust and reliable tool for quantifying carbonyl and dicarbonyl compounds in infant formulae.

The assessment of the environmental impact using the AGREEprep tool has provided valuable insights into the eco-friendliness of the developed HS-SPME-GC-MS procedure for carbonyl and dicarbonyl compound analysis in infant formulae. Notable strengths include reduced hazardous material usage, high material reusability, efficient sample processing, and semi-automation. The method presents a greener alternative compared to existing approaches, emphasizing its potential for sustainable analytical practices in food analysis.

The validated HS-SPME-GC-MS method was employed to detect carbonyl and dicarbonyl compounds in diverse infant formula samples, including powdered and liquid variants. The analysis revealed the widespread presence of compounds like FCHO, GO, and MGO, with varying concentrations, while others like MDA, DMGO, and PhCHO showed more selective occurrence. Significant differences in compound concentrations were found among sample groups, as evidenced by ANOVA results. Furthermore, clustering analyses categorized the infant formula samples into distinct groups based on their carbonyl content, underscoring the potential of these compounds as markers for assessing lipid peroxidation, and food quality.

In conclusion, the presented method stands as a robust tool that can be employed in future research endeavors to investigate the variations in carbonyl content of infant formulae under diverse conditions, providing insights into their association with freshness, product quality, and potential deterioration. Additionally, the presence and quantity of these compounds have significant nutritional and health implications, particularly in the context of infant formulas, making this analysis a valuable tool for evaluating their overall quality, safety, and nutritional impact.

CRediT authorship contribution statement

Jorge A. Custodio-Mendoza: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Ana Lopez Blanco:** Validation, Investigation. **Ana M. Ares-Fuentes:** Writing – review & editing, Writing – original draft, Investigation. **Antonia M. Carro Díaz:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

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

Data will be made available on request.

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Appendix A. Supplementary data

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