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Headspace gas chromatographic method for determining volatile compounds in infant formulas[☆]

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Abstract

Powder infant milk formula quality deterioration and consequently the termination of shelf life results in the appearance of off-flavors mainly determined by a composite effect of spoilage volatiles. A headspace gas chromatographic method to determine propanal, pentanal and hexanal as the main volatiles present in the headspace of powder infant formula oxidation is described as a rapid indication of oxidative status. Under optimum conditions the limits of detection for propanal, pentanal and hexanal were 17.19, 16.87 and 19.60 ng and the limits of quantification were 37.37, 31.96 and 35.97, respectively. The calibration graphs of the method were linear from 25 to 1500, 20 to 3500 and 30 to 8500 ng for propanal, pentanal and hexanal, respectively, with determination coefficients exceeding 0.99. The precision results showed that the relative standard deviations of the repeatability and reproducibility were between 2.2 and 5.5%. The analytical method was simple, rapid, and reliable and permitted the analysis of a large number of formulas using small sample volumes.
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1. Introduction

The period between processing/packaging and the time when milk becomes unacceptable for consumption is called its "shelf-life", and reflects its keeping quality [1]. In the case of powder infant formulas, flavour detection is governed, to a large extent, by fat oxidation [2]. Indeed, lipid oxidation is well recognized as a major cause of quality deterioration during processing or the storage of lipid-rich foods. During peroxidation of unsaturated fatty acids, hydroperoxides are formed and these primary products rapidly decompose to form a complex mixture of secondary lipid oxidation products (alkanes, alkenes, aldehydes, ketones, etc.). Various sampling techniques for aroma isolation and concentration have been reported, such as mass spectrometry-based electronic nose, solid-phase microex-

traction gas chromatography–mass spectrometry, and steam distillation [3–5].

On the other hand, the headspace technique [6–8], which requires minimal sample treatment and reduces artifactual volatile formation, has shown to be an easier, more rapid, and reliable method for the determination of the composition of volatile compounds. In the headspace method, the extraction times and the volumes of sample are less than when using the other methods mentioned previously.

Headspace is a technique used for the analysis of volatiles in food products such as oils [9–14], breast and fish [15–18] and milks [19–27]. The headspace methods extracted a greater number of volatile compounds from samples than did solid-phase microextraction method [28].

Powder milk formulas are rich in polyunsaturated fatty acids such as $n-6$ arachidonic acid (AA) (C20:4, $n-6$) and $n-3$ docosahexaenoic acid (DHA) (C22:6, $n-3$), that are much more susceptible to oxidation than linoleic acid (C18:2, $n-6$), but the oxidation of the latter is also important because is the majority polyunsaturated fatty acid in these milk formulas [29,30]. Moreover DHA produces flavors and odors that are more objectionable. Pentanal and

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hexanal are the specific volatile oxidation products of $n − 6$ polyunsaturated fatty acids and propanal is a product of $n − 3$ polyunsaturated.

The purpose of this study was to develop a technique to identify and quantify the propanal, pentanal and hexanal from oxidized and no-oxidized powder infant formulas. The system proposed is a static headspace gas chromatographic (SHS-GC) method.

2. Experimental

2.1. Samples

A formula rich in polyunsaturated fatty acids (PUFAs) was selected for investigation. It was supplemented with arachidonic acid (C20:4, $n − 6$) and docosahexaenoic acid (C22:6, $n − 3$).

The powder formula was performed over a short period of time (4 weeks) in order to identify potential early oxidation markers. It was stored at 50 °C and analyzed at 0, 3, and 4 weeks.

2.2. Reagents and standards

Stock standard solutions were prepared by dissolution of volatile compounds in Milli-Q water; and stored at 4 °C. The Milli-Q water was purified by passing through a Compact Milli-Q water system from Millipore (Bedford, MA, USA). The propionaldehyde standard with 97% pure, the valeraldehyde standard with 97% pure, and the butyl acetate with 99.7% pure as internal standard were obtained from Aldrich (Steinheim, Germany). The hexanal standard was purchased from Sigma (St. Louis, MO, USA).

2.3. Assay procedure

2.3.1. Headspace analysis procedure
Exactly 0.05 g of powder infant formula was weighed into special 10 ml headspace vials and 2400 µl of Milli-Q water and followed by 600 µl of an internal standard solution (0.84 µg/ml of butyl acetate in Milli-Q water) were added. The vial was sealed with silicone rubber PTFE caps by using a crimper. Immediately, the samples were equilibrated at 60 °C for 15 min with the 2t® Vial Heater Model VH 6200 from Tracer (Teknokroma, Barcelona, Spain). The sampling time (which was 30 s) was measured with a 2t® Static Headspace Sampler MHS 123 from Tracer.

Five hundred microlitres volume of the sample was injected and the volatile compounds were identified by comparison of their peak retention times with those of the standards.

2.3.2. Gas chromatographic analytical conditions
Propanal, pentanal and hexanal were separated in less than 10 min using a Shimadzu Model GC-14 A gas chromatograph equipped with a flame ionization detector, a split–splitless manual injector and an integrator Shimadzu Model C-R6 (Shimadzu, Kyoto, Japan). A fussed-silica capillary column was employed, Supelcowax-10 (bonded, Carbowax 20 M polyethylene glycol) 60 m × 0.32 mm i.d., 0.25 µm film thickness, supplied by Supelco (Bellefonte, PA, USA). The aldehydes were separated isothermally at 75 °C. The injector and the detector temperatures were 185 and 200 °C, respectively, with a split ratio of 1:20. Helium was used as a carrier gas at a linear velocity of 26.02 cm s^{-1}.

3. Results and discussion

3.1. Preparation of sample

The influence of parameters that potentially affected the extraction process (equilibration and sampling time, equilibration temperature and dilutions with Milli-Q water) was studied in order to establish the optimal conditions for maximum recovery of volatile compounds and minimum appearance of interferences. The profiles of volatile compounds were obtained using a modification of the conditions of the method described by Ulberth and Roubicek [2].

The volatile compounds content was analyzed at different equilibration temperatures, at 60, 75, 80, 85 and 90 °C, keeping equilibration time (10 min), sampling time (30 s) and amount of sample (0.5 g with 3 ml Milli-Q water). Under these conditions, the content of volatile compounds was constant over the range 60–80 °C. We selected the lower temperature (60 °C).

When the equilibration temperature was optimized, different values of equilibration time were analyzed (10, 15, 20 and 30 min), using the same sampling time and the same amount of sample than previously. The content of volatile compound was the same at 15, 20 and 30 min, and it was smaller at 10 min, so the optimum equilibration time was 15 min.

We also proved different sampling time (10, 20, 30 and 40 s) keeping other parameters constants. We did not observed differences between them, and we selected 30 s because manufacturer of the 2t® Static Headspace Sampler MHS 123 recommended it.

Finally, the influence of dilution of the sample with Milli-Q water was studied with equilibration temperature, equilibration time and sampling time of 60 °C, 15 min and 30 s, respectively. In this method the powder milk samples were analyzed using nine different dilutions with Milli-Q water. The dilution range was from 0.5 g powder milk with 1 ml of Milli-Q water to 0.02 g with 3 ml (Fig. 1). The propanal content was constant in the different dilutions. On the contrary, the hexanal content increased markedly until the dilution of 0.05 g infant formula with 3 ml Milli-Q water, and the content decreased for more diluted solutions. The pentanal content increased slightly until the dilution of 0.20 g infant formula with 3 ml Milli-Q water, and the
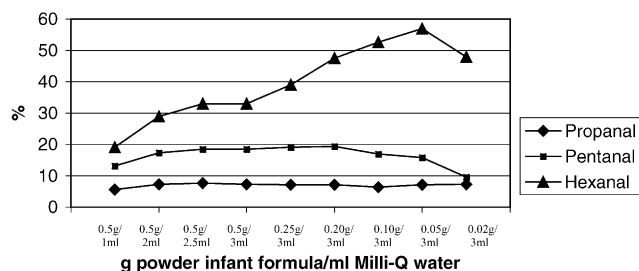
Fig. 1. Volatile compounds obtained in different dilutions of powder formula with Milli-Q water.

content decreased slightly for more diluted solutions. In this way, the optimum dilution was 0.05 g of powder formula with 3 ml of Milli-Q water (2400 μl Milli-Q water and 600 μl internal standard solution) because the majority volatile (hexanal) presented the maximum in this dilution, and the reduction of the pentanal was only 3% from the dilution of 0.20 g formula with 3 ml Milli-Q water to 0.05 g with 3 ml.

3.2. Validation of the chromatographic method

3.2.1. Sensitivity

The detection and the quantification limits were calculated according to the USP criteria [31] by analyzing a number of blank samples and calculating the standard deviation of the background response. The standard deviation multiplied by a factor 3 and 10 provides an estimated of the limit of detection and limit of quantification, respectively (Table 1).

3.2.2. Linearity

For quantitative analysis of volatile compounds, the calibration graph was by linear regression. A constant amount of internal standard (0.84 μg butyl acetate/ml Milli-Q water) was added to increasing analyte concentrations. The peak-area ratio between propanal, pentanal, hexanal and butyl acetate (y) versus the standard mass of propanal, pentanal and hexanal (x) under these conditions was linear over the range 25–1500 ng of propanal, 20–3500 ng pentanal and 30–8500 ng hexanal (Table 1).

3.2.3. Precision

Precision of the method was expressed as the relative standard deviation (R.S.D.) of replicate measurements. The repeatability was established by injecting the powder milk solution six times. The reproducibility was determined by analyzing each sample of powder formula on 6 different days. Table 1 shows precision results. These results met the acceptable precision standards proposed by Horwitz [32] for analyte concentrations with a range 5–40 ppm for the three volatiles studied.

3.2.4. Recovery

Analyzing a sample five times and comparing the analytical result to the known added value showed the accuracy of the method. For estimating the volatile compound recovery, powder milk samples were spiked with propanal, pentanal and hexanal at two fortification levels, 25 and 100% of the estimated initial volatile compound amount. Table 2 shows the recoveries of propanal, pentanal and hexanal after applying the headspace GC method.

The recovery percentages were satisfactory for three volatiles compounds. The results obtained were within the acceptable range of 87 and 109%.

3.3. Application

Propanal, pentanal and hexanal were identified and quantified in non-oxidized and oxidized powder infant formula samples by comparison of their peak retention times with those of the standards. Fig. 2 shows the chromatogram of standards, the retention times were 4.3, 5.6, 7.0 and 7.3 min

Table 1
Sensitivity, linearity and precision of the proposed method

	Propanal	Pentanal	Hexanal
Sensitivity			
Detection limit (ng)	17.19	16.87	19.60
Quantification limit (ng)	37.37	31.96	35.97
Linearity ($y = ax + b$)			
a: intercept	−0.0007	−0.0194	+0.0567
b: slope	0.8193	1.0956	1.0096
r^2: determination coefficient	0.999	0.999	0.999
Precision			
Repeatability[a]	5.63 ± 0.25	8.97 ± 0.34	39.26 ± 0.90
R.S.D. (%)	4.51	3.86	2.29
Reproducibility[b]	5.54 ± 0.30	9.04 ± 0.42	39.41 ± 1.99
R.S.D. (%)	5.51	4.68	5.05

y is area of volatile compound/area of internal standard; x is amount of volatile compound (mcg).
[a] Repeatability expressed as mean (mg kg^{-1}) ± standard deviation.
[b] Reproducibility expressed as mean (mg kg^{-1}) ± standard deviation.

Table 2
Recovery of volatile compounds added to infant formula

Volatile compounds	Amount in formula[a]	Amount added[b]	Amount founded[c]	Recovery (%)	Mean recovery (%)
Propanal	3.58 ± 0.25	128	6.16 ± 0.31	107.18 ± 5.29	109.02
	3.58 ± 0.25	40.9	4.68 ± 0.28	110.87 ± 5.50	
Pentanal	7.68 ± 0.42	332	11.38 ± 0.52	88.27 ± 5.69	87.92
	7.68 ± 0.42	72.8	7.72 ± 0.55	87.58 ± 6.34	
Hexanal	27.38 ± 0.67	1484	47.25 ± 2.74	93.59 ± 3.38	90.86
	27.38 ± 0.67	424	30.00 ± 1.68	88.14 ± 5.34	

[a] Values are expressed as mean $(\text{mg kg}^{-1}) \pm$ standard deviation.
[b] Values are expressed as mcg.
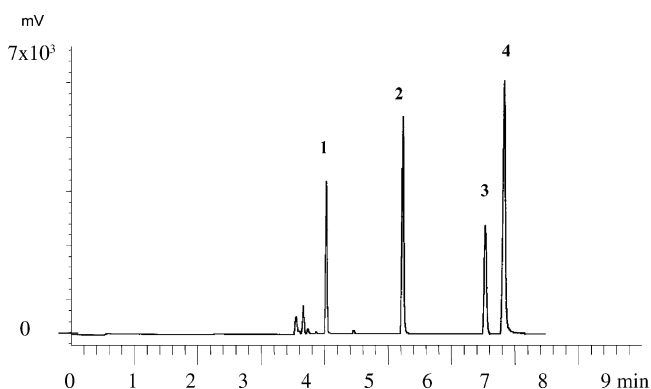[c] Values are expressed as mean $(\text{mg kg}^{-1}) \pm$ standard deviation.

Fig. 2. Chromatogram of standards. Peaks numbered from 1 to 4 correspond to 1, propanal; 2, pentanal; 3, butyl acetate (IS); 4, hexanal. Refer to text for HS-GC conditions.

for propanal, pentanal, butyl acetate and hexanal, respectively.

The oxidized formulas had been stored for over 4 weeks at 50 °C, and analyzed at 0, 3 and 4 weeks. The amount of volatiles was different in these formulas submitted to the static headspace GC method. This procedure gave propanal, pentanal and hexanal amounts ranging from 1.4, 1.5 and 4.6 mg kg^{-1}, respectively, for the relative sample at 0 weeks, up to 5.6, 8.9 and 39.8 mg kg^{-1} for an oxidized one (Table 3). Therefore, the concentrations of volatiles already present in oxidized milk formulas increased markedly, respectively, non-oxidized sample. Fig. 3 shows the volatile compounds profile obtained by analyzing an infant formula

non-oxidized and oxidized (4 weeks of storage) sample. Volatile compound amounts are very dependent on milk powder formulations and storage conditions. On the basis of these results, propanal, pentanal and hexanal could be used to monitor changes in infant formula oxidation during storage. Because of the high level of propanal, pentanal and hexanal found in oxidized samples and its already observed correlation with off-flavors development in milk products [2], these aldehydes were preferred as potential chemical markers to evaluate infant formulas oxidation using the SHS-GC method.

Table 3
Volatile compounds[a] in infant formula analyzed by HS-GC

Volatile compounds	0 weeks	3 weeks[b]	4 weeks[c]
Propanal	1.44 ± 0.06	3.58 ± 0.19	5.63 ± 0.25
Pentanal	1.51 ± 0.05	7.68 ± 0.42	8.97 ± 0.34
Hexanal	4.66 ± 0.21	27.38 ± 0.67	39.84 ± 0.90

[a] Values are expressed as mean $(\text{mg kg}^{-1}) \pm$ standard deviation.
[b] Infant formula stored 3 weeks at 50 °C.
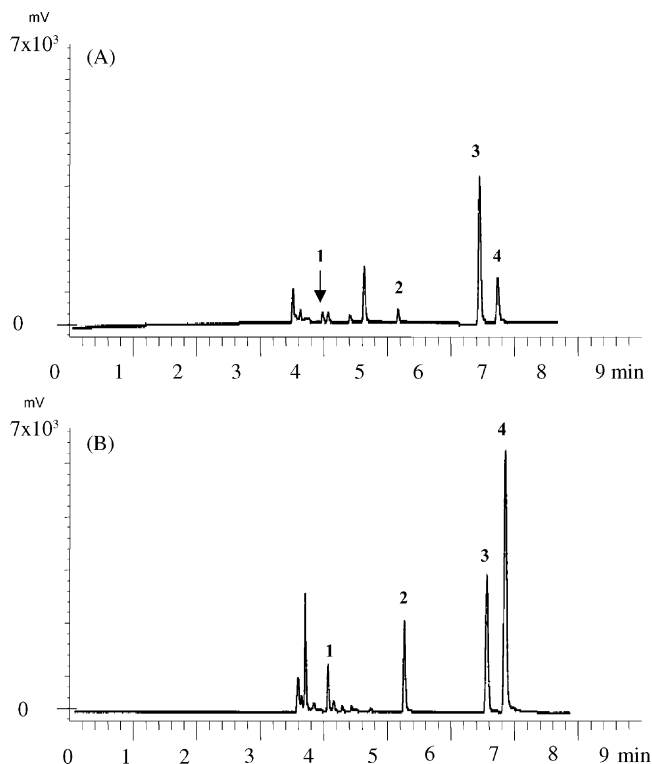[c] Infant formula stored 4 weeks at 50 °C.

Fig. 3. Chromatograms of volatile compounds in infant formula at 0 weeks (A) and after 4 weeks (B). Peaks numbered from 1 to 4 correspond to 1, propanal; 2, pentanal; 3, butyl acetate (IS); 4, hexanal. Refer to text for HS-GC conditions.

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4. Conclusions

The presented results suggest that the proposed method is a reliable, reproducible, and sensitive technique for detecting volatiles in powder infant formulas. The near-absence of sample preparation and the easy-to-use aspect of this technique has potential applications in the food industry as a powerful quality control tool.

Acknowledgements

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