Analysis of volatile compounds in powdered milk for infant nutrition by direct desorption (CIS4-TDU) and GC-MS

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Abstract

Direct thermal desorption coupled with the Gas Chromatography-Mass Spectrometry (TDU-CIS4-GC-MS) technique applied to powdered milk was used as a novel approach for the characterization and quantification, as relative abundance, of volatile organic compounds (VOCs). The aim of this study was to exploit the potential applications and setup conditions of the CIS4-TDU-GC-MS technique for the identification of oxidized or non-oxidized samples of powdered milk for infant nutrition, subjected to accelerated aging through the changes in their VOCs profile over time. Thermal desorption at 30°C and subsequent cryotrapping at –40°C with a Gerstel Liner–in–Liner system, allowed a direct thermal desorption and cryotrapping of VOCs without any memory effect, thus avoiding sample preparation and contamination. The analyses led to the identification of several characteristic off-flavors in the oxidized samples, which were used as molecular markers to discriminate samples with different

oxidation degrees. Finally, VOC contaminants possibly present in the packaging can also be identified with this technique.

Keywords: food analysis; lipid oxidation; baby food; laboratory test; solid sample

1. Introduction

The composition of the aromatic components is a key factor in establishing the quality, the typical source, and the value of foodstuffs, from both an economical and/or organoleptic standpoint. Food products are generally composed of a large number of volatile compounds, the concentrations of which may be very different [1, 2]. The alteration of food sensory properties, which may easily be perceived by the consumers, is mostly caused by the formation of volatile compounds due to degradation and lipid oxidation reactions [3]. Long-Chain Poly Unsaturated Fatty Acids (LC PUFA) refined from natural oils are frequently added to foodstuffs to confer a positive health effect [4, 5, 6]. Since LC PUFA are rather unstable and prone to oxidation, the quality of these oils and fats must be controlled. Some aldehydes, ketones and other compounds are markers of oil and fat quality (Figure 1) [7, 8], since they have unpleasant odors and/or tastes (e.g. fishy) that are not acceptable to consumers. The compound 4-heptenal, for example, adds a detectable fishy odor and taste to food products at concentrations as low as 10 ng g-1 [9]. The GC-MS (Gas Chromatography-Mass Spectrometry) technique applied to volatile organic compounds (VOCs) allows for their characterization and quantification, as relative abundance, in food samples [10, 11, 12]. However, VOCs sampling represents the most critical point in this approach [13]. The majority of odor compounds are volatile, and generally easy to extract from the sample, using various, well-established techniques. The challenge facing the analyst is that of separating these compounds from the sample matrix of a product without analytic discrimination, so that the reconstituted flavor pattern matches the one experienced by the consumer. Sample preparation must therefore ensure sample homogeneity, convenient sample handling, and, possibly, automation. Consequently, while qualitative analyses are generally simpler in solid samples compared to liquid ones (due to the lack of a large liquid compartment where the analytes are solubilized or dispersed) and the signals are more intense if the concentrations are equal (on weight/weight basis), the quantitative approach is far more complicated. Even in the presence of careful sample homogenization and milling, conveniently achieved using an ultra-turrax blender in temperature-controlled baths or with the aid of liquid nitrogen during grinding, a different porosity (in the case of aggregate particles), or stacking of individual nonporous particles, may affect the diffusion of volatile molecules and kinetically influence the solid–headspace equilibrium [13].

The aim of this study was to investigate the innovative application of direct thermal desorption-cryofocalization on VOCs sampling, achieved with Gerstel liner-in-liner thermal desorption and cryofocalization coupled with the gas chromatography-mass spectrometry (TDU-CIS4-GC-MS) technique, for both identification and comparison of the VOCs profile in samples of powdered milk for infant nutrition subjected to accelerated aging.

2. Experimental method

2.1 Test samples

Specimens of powdered milk, produced in 2012 and 2013 and provided by HEINZ ITALIA S.P.A. (Milan), were analyzed. In particular, eighteen samples of Infant Formula, Follow On Formula, Toddler Milk and Pregnant Mother Formula were examined, as reported in Table 1 [14]. The production process consisted of a mixed wet and dry blending process, and two types of dry blending were used, indicated as X and Y in Table 1. The powdered milk samples had a shelf life ranging from 0 to 13 months at the time of the first GC-MS analysis. The GC-MS analyses were performed at regular time intervals until the oxidation was perceived and afterwards on selected samples only.

2.2 Sampling

Since powdered-milk packaging at the consumer scale is carried out with 500-g specimens, the experiment setup would have required too large a sample amount to be conveniently managed at the lab scale. Therefore, a smaller quantity of samples (50 g) were packed at the Plasmon S.p.A. factory (Ozzano Taro, PR) under the same commercial packaging conditions, such as proportions of the product and head-space. In particular, the oxygen was replaced with nitrogen flushing up to have <3% of O_2 in the head-space and then sealed. For each product, eighteen samples were packed in composite laminated bags (CLB) provided by the Company, material structure PET 12 µm/PA 15 µm/ALU 9 µm/PP 70 µm. The sample bags were then distributed according to the aging protocol, illustrated below, i) in an oven at 40° C, and ii) at room temperature.

2.3 Accelerated aging protocol

In order to evaluate the stability of the sample of milk powder in a short time, an accelerated aging protocol was followed as reported in the International Conference on Harmonisation (ICH) guidelines [15, 16]. The samples were sealed in plastic bags, which were randomly placed in a static oven at 40°C±2. Due to the possibility of contact with the oxygen favoring the formation of oxidation byproducts, individual sample bags were prepared for each sampling time, in order to avoid bag opening and re-sealing after each sampling. One sealed bag for each sample was withdrawn from the oven at each sampling time (every 10 days) and immediately analyzed.

2.4 TDU-CIS4-GC-MS conditions and VOCs characterization

The VOCs profile of powdered milk was determined by thermal desorption at 30°C, with cryotrapping at -40°C of the stripped VOCs in a Programmable Temperature Vaporizer (Gerstel Cooling Injector System, CIS4) injector, and analyzed by GC-MS. A tentative compound identification was performed by comparing the Mass spectra of each peak against those reported in mass spectral databases after Dynamic Background Compensation by Clear View software, (ALMSCO, UK).

For thermal desorption, 100 milligrams of powdered milk were placed in a thermal desorption unit (TDU) liner provided with a microporous sinthered glass and supplemented with 11.1 µL of a 1 mg L^{-1} solution of hexanoic acid-d11 in pentane as the Internal Standard (IS) using a calibrated syringe. Since the gas stripping flow may be affected by differences in the sample homogeneity and granulometry, thus influencing the peak area of the analytes, the Internal Standard was placed on top of the powdered milk and used for normalizing the analyte responses over the area of the IS.

The samples were immediately analyzed by CIS4-TDU-GC/MS. Gerstel TDU was heated at 30°C for 30 min under a helium stripping flow of 20 ml min^{-1}. The TDU unit was directly assembled over the PTV injector (CIS4 Gerstel, Germany) with a Liner–in–liner coupling which eliminates any loss of analytes and the carryover effect. During this stage, the CIS4 was cooled to -40°C by computer controlled liquid CO_2 pulsed flow. After cryo-trapping, the PTV was quickly ramped to 260°C for desorption and the analyte transferred to CIS4. An Agilent 7890 GC equipped with a 5975 MSD was used for the analysis equipped with an Agilent DB WAX 50 m, 0.20 µm id, 0.40 µm df. The

chromatographic conditions were: initial temperature 40°C, then 10°C min^{-1} up to 260°C, then hold for 6.6 min.

2.5 Sensory analysis

Samples were followed by performing an in-factory sensory evaluation during aging. Panel members tested samples 'as is' and after reconstitution with water. The sensory analyses were performed at room temperature. The samples (100 g and 100 mL reconstituted as reported on the label) were presented to the sensory panel in glass vessels (capacity 140 mL) for the organoleptic evaluations. In particular, a panel composed of 5 members collaborated on the sensory assessment. The panelists were asked to score the intensities of selected attributes on a scale from 0 (no perception) to 5 (strong perception). These attributes were specifically: rancid, metallic, oxidized. For the color evaluation a reference scale from withe to yellow was considered.

2.6 Statistical analysis

The peak areas relating to the tentatively identified compounds were normalized over the area of the IS in each chromatographic run and analyzed by the student's T test between the oxidized and non-oxidized samples as classified by the sensory analysis. Systat 12 (Systat Software, Inc. Chicago, IL. USA) was used for the computation.

3. Results

3.1 Packaging analysis

Since polymeric plastic materials used in packaging may contain stabilizers [17], a preliminary check was made of the plastic bags to ascertain the presence of these compounds in the form of preservatives able to contaminate the powdered milk. Two polymeric materials, i.e. polythene and CLB packaging, were analyzed using the same analytical conditions as the infant formula. The polythene envelope contained two isomers of dipropylen glycol butyl ether (MW 190.16), (RT of 16.8 and 16.95 respectively), an intermediate chemical solvent used in the plasticizer manufacturing process. The peak at RT 19.8 corresponded to the antioxidant Butylated Hydroxytoluene (BHT) (see supplementary

file, Figure 1S). Conversely, while the CLB bags did not contain any of the solvents or antioxidants present in the other kind of bag, the MS profile showed the presence of characteristic products due to contamination from the operator's hand including Limonene, Hexadecenoic acid, etc. In addition, N-butyl-Benzensulfonammide was identified as a contaminant, presumably only present on the outer part of the envelopes (colored label). Since in a preliminary experiment, the analysis of the powdered milk contained in these envelopes did not show either the volatile compounds from bare-hand contamination, or N-butyl-Benzensulfonammide, a potentially toxic molecule, the envelopes used were deemed suitable for packaging the samples subjected to accelerated aging.

3.2 Powdered milk composition and analysis

The samples considered in this study were characterized by the nutritional composition reported in Table 2. Infant Formula, Follow On Formula and Toddler Milk showed a mean of 22.6 % of fats and Pregnant Mother Formula of 8.0 %. Linoleic acid was the most abundant compound among the ω-3 fatty acids, followed by α-Linolenic acid, Arachidonic acid and DHA. The carbohydrate content was similar for all products while the protein content was higher in the Pregnant Mother Formula samples, 26% compared to a mean of 14.3% in the Infant Formula, Follow On Formula and Toddler Milk samples.

The presence of volatile carbonyl compounds generated from the decomposition of lipid hydroperoxides is an established indicator of lipid oxidation products in some food systems. Most of these compounds are usually aldehydes, the smell of which can be defined as pungent, "green (or herbaceous)", "fat", "food-fried" etc. [3, 10]. Hexanal, 2-(E)-Hexenal, 4-(Z)-Heptenal, 2-Pentylfuran, 1-Octen-3-one, 2,4-(E,E)-Heptadienal, 2,6-(E,Z)-Nonadienal, 2,4-(E,E)-Nonadienal, 2,4-(E,E)-Decadienal are typical fatty acid degradation compounds [18].

The oxidized samples, as identified by sensory analysis, showed the presence of volatile compounds (mostly aldehydes) associated with the product oxidation, i.e.: Pentanal, Hexenal, Octanal, 2-Heptenal, Nonanal, 2-Octenal, Furfural, 2-Nonenal, 2-Decenal, 2,4-Nonadienal, 2-Undecenal, 2,4-Decadienal, 2-Furanmethanol, Hexanoic acid and Pentanoic acid (Figure 1, Table 3).

Sample 16 was subjected to induced oxidation due to unsuitable storage conditions (3 weeks at 45°C, 80% RH-relative humidity) and selected to verify the causes and indicators comparable with those detectable in the non-compliant Sample 5. Samples 16 and 5 (Figure 1A) showed the presence of comparable amounts of both volatile compounds and aldehydes associated with the product oxidation. Sample 2 has been reported to compare the previous samples with a compliant sample, (Figure 1 B). The profile of Sample 2, which confirmed its good state of conservation, showed a lower number of peaks than the previous samples, and their lower relative abundance in terms of intensity.

The statistical analysis conducted on the entire dataset of milk with different formulas, different shelf-life and different storage temperatures, revealed significant differences between oxidized and non-oxidized samples after Student's T test for most of the monitored compounds (Table 3). 2-Heptenal, 2-Octenal, 2,4-Nonadienal, 2,4-Decadienal isomer 1, 2,4-Decadienal isomer 2, 2-Undecenal, Hexanal, 2-Furanmethanol, Pentanoic acid, Hexanoic acid, Octanal, Nonanal, 2-Nonenal resulted significantly different albeit at different probability level. On the contrary, 1-hydroxy-2-Propanone, Furfural, Decanal, 2-Decenal were not significantly different between oxidized and non-oxidized samples at $p \leq 0.05$. The sum of aldehydes was also calculated and was significantly higher for oxidized samples at $p=0.010$.

However, the off-flavor concentration continued to rise with aging, therefore, in order to detect the compounds responsible for the initial oxidized smells, a statistical analysis was repeated on a reduced dataset where only the non-oxidized specimens or those classified as oxidized for the first time were included (Table 3). Surprisingly, the majority of the aldehydes resulted not significantly different between the two groups, mainly due to the large data dispersion around the mean value. This is most probably the result of an initial non-homogeneous shelf-life at the beginning of the experiment.

3.3 Selection of indicator compounds for product decay in accelerated aging conditions

The technique adopted allowed the evaluation of the oxidative grade of milk samples at different aging. The shelf-life of properly stored, unopened containers of powdered milk for infant nutrition, guaranteed by manufacturers is 24 months, which implies that completion of the oxidation process would take several years. The ideal test for measuring the changes in powdered milk samples during

storage would be a test in which the milk is stored under realistic storage conditions and the changes are regularly evaluated over time. An obvious drawback of such an approach is that it takes a very long time. On the basis of the manufacturers' guidelines, optimized by analysis of selected nutrients, peroxides and estimation of color preservation, the accelerated aging protocol assimilates the storage of one month at room temperature to 10 days of storage at 40°C in an oven. The accelerated storage conditions were such to resemble the oxidation mechanism and avoid artifacts as reported in experimental section. The presence of metal ions, exposure to irradiation (normal or UV light) and very high temperatures are therefore not acceptable [19, 20]. This approach also eliminates any differences in parameters such as temperature and head space-to-powder volume ratio. The aim of this work was to evaluate the performance and applicability of TDU-CIS4-GC-MS, which, using accelerated ageing conditions, permit food stability to be investigated. Further investigations are underway for defining the correct correspondence between real-time stability and accelerated stability tests. Selected VOCs monitored in samples of powdered milk for infant nutrition during the accelerated aging protocol are reported in Table 3. During the aging of milk samples, certain marker compounds increase. In order to associate the identified markers with the increase of off-flavors, a sensory analysis of the samples was also conducted at the same time. The histograms in Figure 2 show the compounds identified as markers, comparing normalized analyte responses of hexanoic acid and aldehydes (sum of Hexenal, 2-Heptenal, 2-Octenal, 2-Nonenal, 2,4-Nonadienal, 2-Undecenal, 2,4-Decadienal) observed in selected infant milk samples during aging. Figure 2 reports the area values (indicated with *) corresponding to oxidized samples according to the panel members' scores. This suggests that the hexanoic acid and aldehydes contents can be reliably associated with the off-flavor evaluation.

The technique adopted allowed for a direct comparison of samples with different formulations, technologies and/or packaging.

4. Conclusions

Direct thermal desorption and cryo-trapping of VOCs with liner-in-liner system allows qualitative and semi-quantitative analysis of powdered milk for infant nutrition samples without memory effect, and

avoiding sample preparation and contaminations. This simple and innovative technique makes it possible to identify markers of lipid oxidation in infant milk formulas and to monitor them during aging. The VOC analysis proved to be useful for tracking the early stages of lipid oxidation and discriminating milk samples at different degrees of oxidation. This analytical approach enables a direct analysis of the packaging material under the same conditions as the samples. The TDU method is suitable for obtaining an overview of the quality and the emission potential of a solid material and therefore, it can be useful in searching for an appropriate packaging material. Finally, this approach could also be applicable with other types of solid food matrices.

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Figure Captions

Figure 1.

Comparison of GC-MS analyses of oxidized (A-sample 16) and non-oxidized (B-sample 2) infant milk formula. IS: internal standard; * aldehydes; 1. Butanoic acid; 2. Pentanoic acid; 3. Hexanoic acid. Zoom of original TIC.

Figure 2.

The duration of storage in months is shown on the x axis, and the y axis shows the normalized areas of compounds as detected by GC–MS. *Days when sample turned to be oxidized for panel members: sample 1: 10 months; sample 2: 8 months; sample 3: 10 months; sample 7: 11 months.

A: sum of Hexenal, 2-Heptenal, 2-Octenal, 2-Nonenal, 2,4-Nonadienal, 2-Undecenal, 2,4-Decadienal

B: Hexanoic acid

Tables

Table1: Powdered milk for infant nutrition samples analyzed in this study.

Sample	Product	Format	Dry Blending Process
1	Infant Formula	Can	X
2	Infant Formula	Can	X
3	Follow On Formula	Can	X
4	Follow On Formula	Can	X
5	Toddler Milk	Can	X
6	Toddler Milk	Can	X
7	Toddler Milk	Can	X
8	Infant Formula	Can	Y
9	Follow On Formula	Can	Y
10	Infant Formula	Bag in Box	Y
11	Follow On Formula	Bag in Box	Y
12	Follow On Formula	Bag in Box	Y
13	Toddler Milk	Can	Y
14	Follow On Formula	Can	Y
15	Toddler Milk	Can	Y
16	Infant Formula	Can	X
17	Pregnant Mother Formula	Can	Y
18	Pregnant Mother Formula	Can	X

Table 2: Lipid profile and relevant macronutrients composition of milk samples.

*of reconstituted powder

	Infant Formula		Follow On Formula		Toddler Milk		Pregnant Mother Formula	
	100 g	100 ml at 13.1%*	100 g	100 ml at 14.1%*	100 g	100 ml at 16.9%*	100 g	100 ml at 16.9%*
Energy (kJ)	2097	275	1997	282	2000	338	402	69
Protein (N x 6.25) (g)	11.0	1.44	15.5	2.2	16.5	2.8	26.0	4.5
Carbohydrate (g)	54.3	7.1	55	7.8	54	9.1	54.0	9.3
Fat (g)	26.0	3.4	21.0	3.0	21	3.5	8.0	1.4
• Linoleic acid (mg)	4555	597	3800	536	3800	642	1650	284
•α-Linolenic acid (mg)	472	62	630	89	630	106	220	38
• Arachidonic acid (mg)	140	18.3	110	15.5	24	4.1	-	-
• DHA (mg)	70	9.2	55	7.8	12	2.0	60.0	10.3
GOS (galacto-oligosaccharides) g	3.1	0.4	2.8	0.4	2.4	0.4	3.0	0.5

Table 3: Selected VOCs monitored in infant formula samples during accelerated aging protocol.

Compound	RT	p-value*	Mean oxidized§	Mean non-oxidized§	p-value**
Hexanal	08.48	0.014	0.005	0.001	0.083
Octanal	11.58	0.040	0.016	0.021	0.386
2-Heptenal	12.35	0.001	0.006	0.001	0.112
1-hydroxy-2-Propanone	12.48	0.260	0.014	0.031	0.100
Nonanal	13.12	0.044	0.060	0.049	0.494
2-Octenal	13.73	0.001	0.008	0.001	0.041
Furfural	14.47	0.094	0.003	0.004	0.311
Decanal	14.60	0.747	0.018	0.041	0.102
2-Nonenal	14.75	0.046	0.002	0.000	0.098
2-Furanmethanol	16.54	0.018	0.006	0.005	0.715
2-Decenal	16.56	0.383	0.017	0.014	0.767
2,4-Nonadienal	17.31	0.001	0.071	0.001	0.175
Pentanoic acid	17.75	0.019	0.018	0.002	0.083
2-Undecenal	17.80	0.006	0.013	0.000	0.015
2,4-Decadienal isomer 1	18.11	0.001	0.164	0.003	0.153
2,4-Decadienal isomer 2	18.56	0.001	0.028	0.001	0.138
Hexanoic acid	18.73	0.010	0.320	0.011	0.022

*Probability level between oxidized and not oxidized samples calculated for the entire dataset

§ Normalized compound responses over IS area, concerning p-value**

** Probability level between oxidized and not oxidized samples calculated for a reduced dataset including oxidized specimens at the first occurrence. See text for details.