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Comparison of analytical techniques to quantify malondialdehyde in milk powders

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Abstract

Several analytical methods were compared to quantify malondialdehyde (MDA) in milk powders. Modified thiobarbituric acid (TBA) methods, using either visible spectrophotometry (direct absorbance reading or after third derivative transformation of the spectrum) or HPLC, required derivatisation at elevated temperature, which appeared to catalyse artefactual MDA formation and thus overestimate the MDA content. In contrast to the TBA derivatisation method, the measurement of MDA as the dinitrophenylhydrazone derivative by HPLC or as the phenylhydrazone product by GC–MS with a deuterated internal standard resulted in lower estimates in the ranges of 2–17- and 3–30-fold, respectively; apparently due to the milder derivatisation conditions. The estimates of MDA determined by both HPLC–UV and GC–MS techniques result in lower values which are similar in magnitude even though the GC–MS technique is more sensitive. Published by Elsevier Science B.V.

Keywords: Food analysis; Lipid peroxidation; Malondialdehyde

1. Introduction

Lipid oxidation is a major cause of quality deterioration during the storage of lipid-rich foods. Hydroperoxides, the primary products of lipid oxidation, are colorless, tasteless and odorless. The breakdown products of these peroxides yield a complex mixture of low-molecular-mass compounds with distinctive odor and flavour characteristics, including alkanes, alkenes, aldehydes, ketones, alcohol, esters and acids [1]. These compounds impart off-flavours and loss of nutrients to food products such as milk powders and thus limit the shelf life of the product [2,3]. Quantification of primary lipid peroxidation products (hydroperoxides) is difficult due to the unstable and reactive nature of these compounds [4]. Thus, the assessment of lipid peroxidation is usually performed by analysing the secondary oxidation product such as malondialdehyde (MDA). This compound has long been employed as a model compound for studying secondary degradation products of lipid peroxidation. The condensation of MDA with two molecules of 2-thiobarbituric acid (TBA) has been widely used to measure the extent of oxidative deterioration of lipids in biological and food systems [5–7]. The absorbance of the complex is usually measured by spectrophotometry or by spectrofluorometry [8]. However, these methods of

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quantitation are non-specific and may lead to the overestimation of the MDA content [1,9]. Specific techniques, also based on TBA adduct, were therefore developed such as derivative spectrophotometry [10,11] or HPLC separation with spectrophotometric [12] or spectrofluorometric [13,14] detection. However, derivatisation of MDA with the TBA reagent requires elevated temperatures (70°C), and the high-temperature may catalyse artefactual MDA formation in complex food matrices [1,15]. Therefore, other reagents containing a hydrazine function have been employed to measure MDA, enabling derivatisation under milder conditions, which potentially minimises artefactual MDA formation. For example, derivatisation of MDA can be performed with 2,4-dinitrophenylhydrazine with subsequent analysis by HPLC with UV detection [16], LC–MS [17] or by GC–MS [18]. Levels of MDA have also been measured by ion-pairing HPLC using myristyltrimethyl ammonium bromide [19] or sodium phosphate [19–21] in the mobile phase which led to a better chromatographic separation.

The goal of this study is to compare MDA content in milk powders using different analytical methods and to determine the most appropriate technique in terms of potential artefactual MDA formation. These methods include: the TBA tests (quantitation by spectrophotometry, direct absorbance reading and third derivative measurement, and by HPLC); HPLC with UV detection after derivatisation with dinitrophenylhydrazine, and derivatisation of MDA with different hydrazine derivatives using stable isotope dilution GC–MS technique.

2. Experimental

2.1. Chemicals

Ammonium acetate, potassium dihydrogen phosphate and trichloroacetic acid (TCA) were obtained from Merck (Geneva, Switzerland). 2,4-Dinitrophenylhydrazine (DNPH), heptafluoro-*p*-tolylphenylhydrazine (HFPH), phenylhydrazine (PH), 1,1,3,3-tetraethoxypropane (TEP) and thiobarbituric acid (TBA) were purchased from Aldrich (Buchs, Switzerland). Butylated hydroxytoluene (BHT) and pentafluorophenylhydrazine (PFPH) were obtained from Sigma (Buchs, Switzerland). 1-3-d_2-1,1,3,3-Tetraethoxypropane (d_2-TEP) was a gift from Professor L.J. Marnett (Vanderbilt University School of Medicine, VA, USA). All other chemical reagents were of analytical or HPLC grade.

2.2. Preparation of MDA and d_2-MDA standard solutions

TEP (ca. 23 mg) was diluted in HCl 0.1 N (10 ml) and this solution incubated at 40°C for 40 min to hydrolyse TEP into MDA (final concentration ca. 10 m*M*). The concentration of MDA was determined by measuring its absorbance at 244 nm (ε=13 700 in H_2SO_4 1%). d_2-MDA was prepared in a same way from d_2-TEP to obtain a final concentration of 100 μ*M* in HCl 0.1 N. Standard solutions of MDA and d_2-MDA were then prepared by successive dilutions with HCl 0.1 N.

2.3. Milk powder samples

Four different milk powder samples, each fortified with iron, polyunsaturated fatty acids and vitamins C and E were used for the comparison of different analytical methods.

2.4. MDA analysis methods

2.4.1. Spectrophotometric determination of MDA-TBA

Milk powder (5 g) was solubilised in bidistilled water (50 ml final volume) pre-heated at around 40°C. An aliquot of this slurry (1 ml) was transferred into a 5-ml Nunc Cryotube™ (Polylabo, Geneva, Switzerland) followed by successive additions of TBA 1% in TCA 5% (800 μl) and BHT 0.8% in ethanol (800 μl). The mixture was then homogenised and centrifuged at 2700 *g* for 5 min (Centrifuge Mistral 2000, MSE Scientific Instrument, Leicestershire, UK). The upper phase was transferred into a new tube and derivatisation was performed by heating at 70°C for 1 h. After cooling, the solution was analysed by spectrophotometry using a Lambda Bio 20 spectrophotometer (Perkin-Elmer, Rotkreuz, Switzerland) using a 1-cm absorption cell. Spectrum of MDA-TBA solutions was recorded from 400 to 650 nm at a scanning speed of 240 nm/min against

the blank reaction mixture. Absorbance readings were done at 532 nm. Third derivative spectrum was obtained by electronic differentiation ($d^3A/d\lambda^3$, where A is the absorbance and λ the wavelength) of the normal visible spectrum, using a derivative difference ($\Delta\lambda$) of 21 nm. Peak height measurements were performed at 521.5 nm. Data acquisition and processing were carried out with the Perkin-Elmer UV Winlab software. MDA derivative was quantified using an external standard calibration curve.

2.4.2. HPLC determination of MDA-TBA

The extraction procedure was the same as that described for the spectrophotometric determination of MDA. A Hewlett-Packard 1090 HPLC instrument equipped with a diode-array detector (DAD) was used for quantification. The column used was a Beckman Ultrasphere ODS-C_{18} (250×4.6 mm I.D., 5 μm) connected to a Supelco LC-8-DB guard column. The analyses were conducted by injecting 40 μl of sample in isocratic mode with 50 m*M* potassium phosphate (pH 6)/methanol (60:40, v/v) at a flow-rate of 1 ml/min and monitored at 532 nm. Data acquisition and processing were carried out with the Hewlett-Packard HP-Chem software. The MDA derivative was quantitated against an external calibration curve in the concentration range from 17 to 2000 μg/kg of milk powder.

2.4.3. HPLC determination of MDA-DNPH

Milk powder (5 g) was solubilised in bidistilled water (50 ml final volume) pre-heated at around 40°C. An aliquot of this slurry (1 ml) was transferred into a 5-ml Nunc Cryotube™ followed by successive additions of TCA 5% (700 μl) and BHT 0.8% in hexane (700 μl). The solution was first thoroughly vortexed then centrifuged at 2700 *g* for 5 min. The upper hexane phase was removed and derivatisation was done by adding DNPH (100 μl of a 1 m*M* solution in HCl 2 N) to the lower acidic aqueous phase and incubating for 1 h at room temperature. The hydrazone was extracted four times with hexane (1 ml). The hexane fractions were evaporated to dryness under a stream of nitrogen and resuspended in acetonitrile/water (50:50, v/v) (100 μl) before HPLC analysis. The HPLC instrument and column were identical as those described above. The analyses were done by injecting 40 μl of sample in isocratic mode with 50 m*M* ammonium acetate/acetonitrile (55:45, v/v) at a flow-rate of 1 ml/min and monitored at 307 nm. The MDA derivative was quantified using an external calibration curve in the concentration range from 14 to 1000 μg/kg.

2.4.4. GC–MS analysis of MDA-PH

Milk powder (5 g) was solubilised in bidistilled water (50 ml final volume) pre-heated at around 40°C. An aliquot of this slurry (500 μl) was introduced into a 2-ml Eppendorf tube, followed by the addition of d_2-MDA (25 μl of a 10 μ*M* solution, 370 μg/kg final concentration), TCA 5% (700 μl) and BHT 0.8% in hexane (300 μl). After mixing and centrifugation at 8400 *g* for 10 min (Eppendorf 5415 centrifuge), the top hexane layer was discarded. Phenyl hydrazine (100 μl of a 9 m*M* aqueous solution) was added to the acidic phase and the derivatisation was performed at room temperature for 1 h. The hydrazone derivative was extracted once with hexane (300 μl), then washed with the same volume of water and 2 μl of the recovered organic solution analysed by GC–MS. Analyses were done with a Hewlett-Packard 6890 series II gas chromatograph system interfaced with a 5972 series mass selective detector and a 7683 automatic sample injector. The column was a J&W DB-225 (20 m× 0.18 mm I.D., film thickness 0.2 μm) which was coupled to a J&W deactivated fused-silica precolumn (1 m×0.53 mm I.D.). The injector and the GC–MS interface temperatures were set at 250 and 280°C, respectively. The oven program was set at an initial temperature of 50°C for 3 min, then increased at a rate of 25°C/min to 220°C, and held at 220°C for 1 min. Two microliters of each sample were injected in triplicate in the splitless mode with helium as carrier gas (constant flow 40 cm/s). MS acquisition was obtained after a 5-min solvent delay, by electron impact ionisation (70 eV) in selected ion monitoring (SIM) mode by recording m/z 144 and 146 for MDA-PH and d_2-MDA-PH, respectively. For unambiguous confirmation of peak identity, a qualifier ion was also monitored at m/z 117 and 120 for MDA-PH and d_2-MDA-PH, respectively. Data acquisition and processing were carried out with the Hewlett-Packard HP-Chem data system. MDA derivative was quantified using an external calibration curve (after plotting the area ratio versus the amount

ratio) in the concentration range from 10 to 4000 μg/kg. The isotopic purity of d_2-MDA was observed at 95% which was taken into account in our calibration curve.

3. Results and discussion

3.1. Spectrophotometry

Our extraction and detection conditions for MDA-TBA have been adapted from Angulo et al. [8] and Botsoglou et al. [10]. The spectra of the third derivative versus underivatised MDA-TBA normal spectrophotometry are illustrated in Fig. 1. In the third derivative spectrum, the positive peak observed at a wavelength of 521.5 nm and the negative one detected at 544 nm lead to an inflection point at 532 nm, which corresponds to the maximum absorption of the underivatised spectrum.

3.2. HPLC

Fig. 2 represents the HPLC chromatograms of two different milk powders where MDA was derivatised by DNPH and TBA. Under these HPLC conditions, the retention time of the MDA-DNPH was 10.2 min with a maximum UV peak observed at 307 nm, whereas the MDA-TBA was detected at 8.8 min with a maximum absorption found at 532 nm. These two derivatisation procedures provided clean chromatogram profiles; however, the estimates of the MDA contents were different: the estimates of MDA by the DNPH and TBA methods were calculated at 113±2 and 385±10 μg/kg, respectively.

3.3. GC–MS

We also quantitated MDA in milk powder by GC–MS under electron impact ionisation. Several different phenylhydrazine reagents (DNPH, HFPH, PFPH and PH) were used for the derivatisation of MDA to evaluate their MS response and the degree of fragmentation. In all cases, except for the MDA-

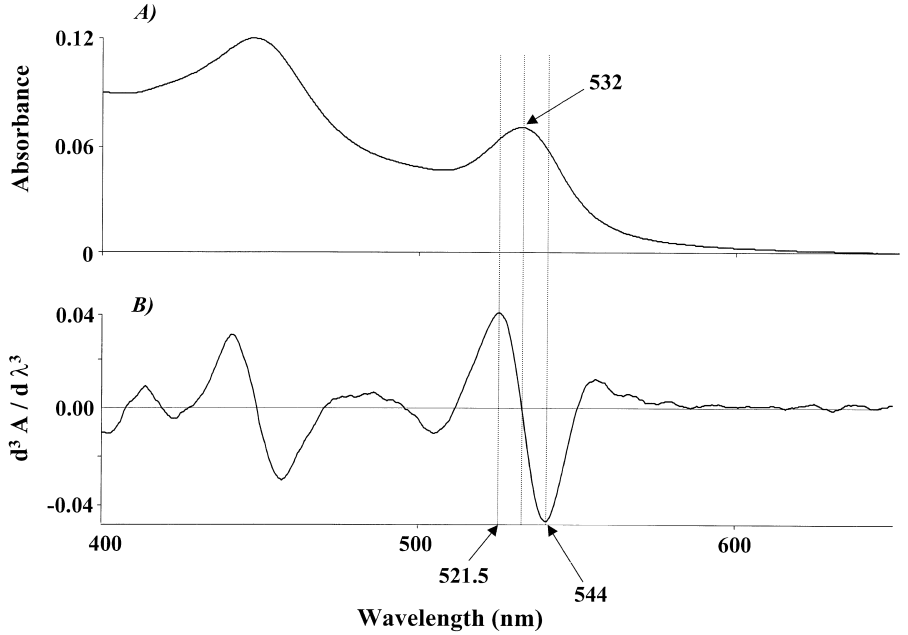

Fig. 1. Visible spectra of milk powder extract derivatised with TBA analysed with (A) normal and (B) third derivative spectroscopy.

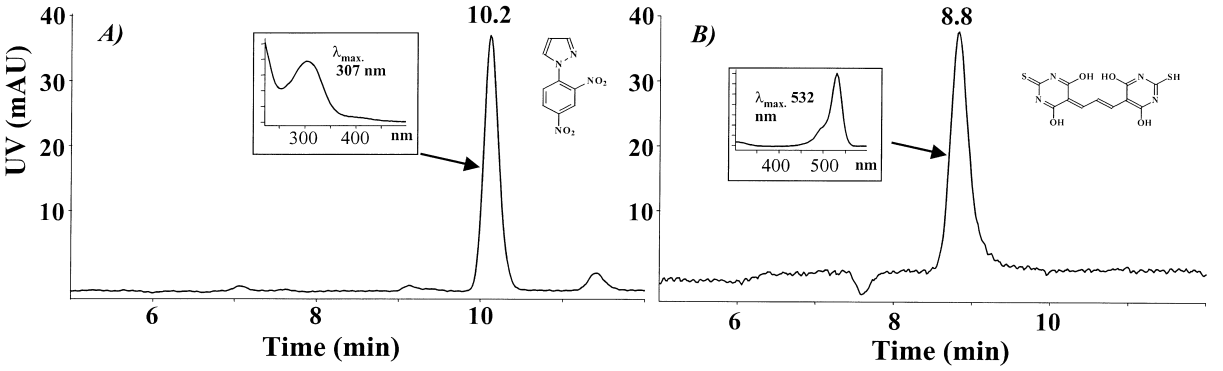

Fig. 2. HPLC chromatograms of MDA in milk powder samples as its (A) DNPH and (B) TBA derivatives.

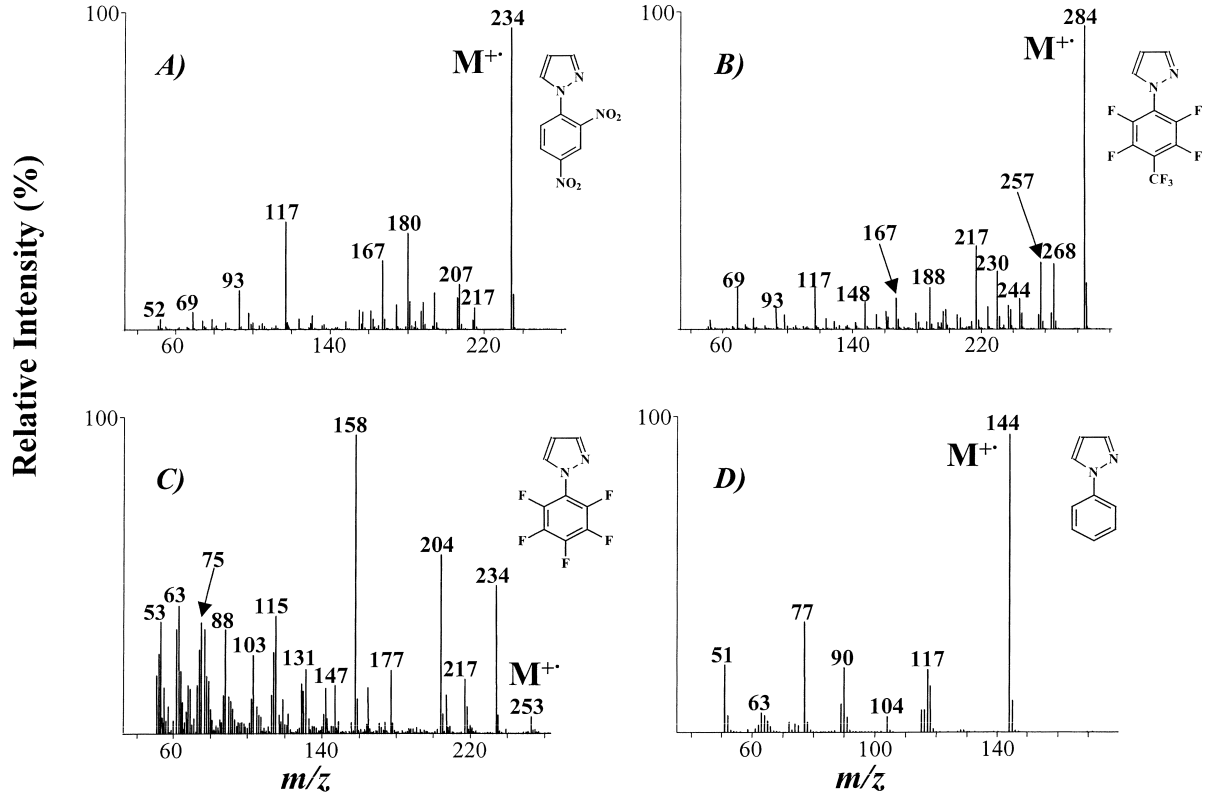

Fig. 3. Full scan electron impact mass spectra of MDA derivatised with (A) 2,4-dinitrophenylhydrazine (DNPH), (B) heptafluoro-*p*-tolylphenylhydrazine (HFPH), (C) pentafluorophenylhydrazine (PFPH) and (D) phenylhydrazine (PH).

PFPH derivative, the molecular ion was observed as the base peak (Fig. 3). The MDA-PH derivative appeared to be most refractory to fragmentation, with the molecular ion accounting for 35% of the total ions. Therefore, MDA-PH was chosen for the quantification of MDA. Fig. 4 represents the total ion current of MDA content, from milk powder extract derivatised with phenylhydrazine and monitored in SIM mode. Under these conditions, MDA-PH eluted at 7.4 min. The utilisation of d_2-MDA as a stable, deuterated internal standard strengthened the identification of MDA due to a nearly identical elution time of both compounds, and enabled quantitative calculation of the analyte.

3.4. Comparison of methods for the quantification of MDA

Table 1 summarises the MDA contents calculated from an unspiked and spiked (at three different levels of fortification) milk powder sample analysed by the different techniques. The higher values obtained with the MDA-TBA classical visible spectrophotometry method (detection at 532 nm) may be explained by the intense interfering peak observed at 450 nm (Fig. 1). This peak corresponds to the absorption of a yellow pigment formed after the TBA derivatisation of milk powders and has been attributed to several other lipid oxidation products such as alkenals, alkadienals, other aldehydes, and ketones [10,22]. Salih et al. [23] reported that spectrophotometric measurements of MDA-TBA could only be made if this absorption band is absent or present at low intensity level and does not overlap with the MDA-TBA peak detected at 532 nm. This interfering band could partly explain the inconsistent recoveries of MDA obtained using this methodology, which is also linked to the poor sensitivity of the direct visible

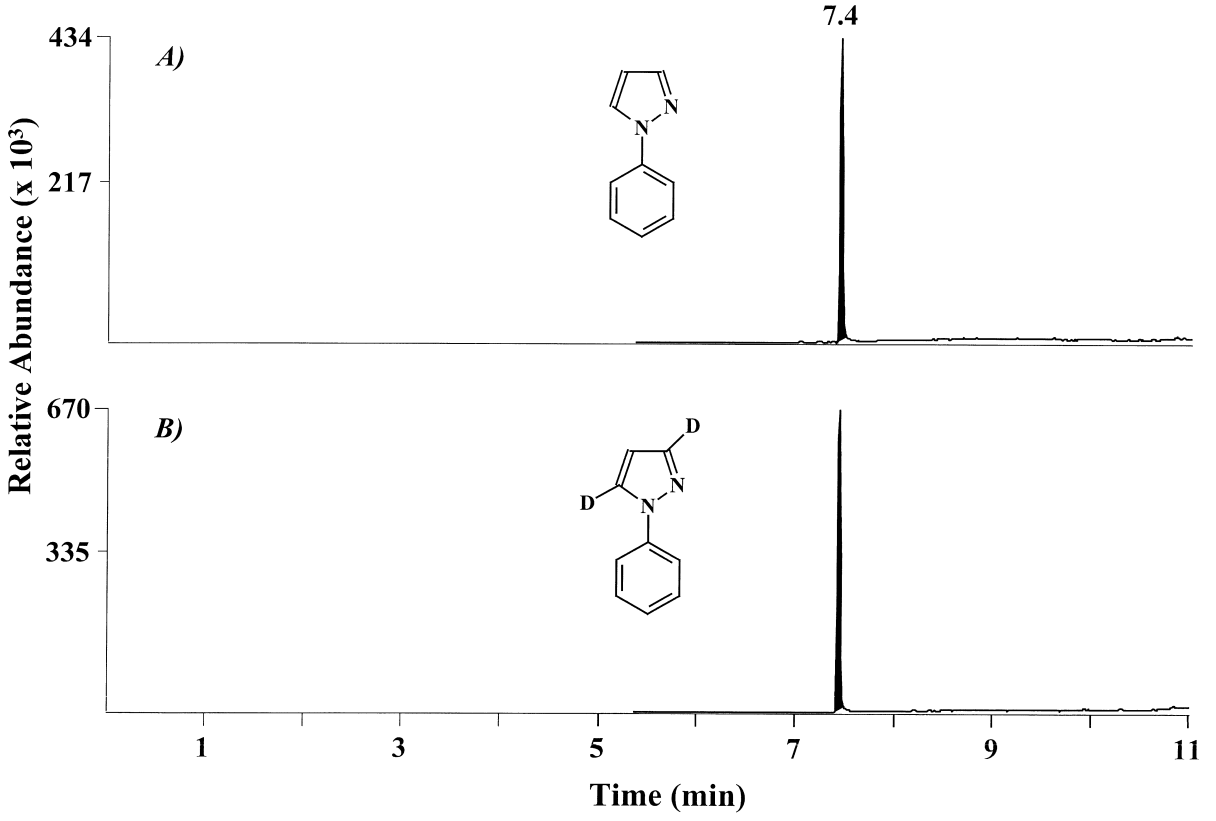

Fig. 4. Total ion current of MDA-PH derivative extracted from milk powder sample analysed by GC–MS acquired in SIM mode by scanning: (A) m/z 144 (MDA-PH) and (B) m/z 146 (d_2-MDA-PH).

Table 1
Precision and accuracy data of MDA estimates in milk powder samples by different analytical methods[a]

Analytical method	MDA added (μg/kg)	MDA found (μg/kg±SD)	RSD (%)	Mean estimated values (%)
Direct visible spectroscopy	0	1022±33	3.2	
MDA-TBA	127	1089±19	1.7	53
	254	1189±23	1.9	66
	381	1313±32	2.4	76
Third derivative spectroscopy	0	684±11	1.5	
MDA-TBA	127	781±4	0.6	76
	254	892±3	0.3	82
	381	984±10	1.0	79
HPLC-visible	0	687±5	0.7	
MDA-TBA	127	784±12	1.5	76
	254	891±13	1.4	80
	381	1017±6	0.6	87
HPLC–UV	0	327±4	1.4	
MDA-DNPH	128	433±13	3.3	83
	256	508±13	2.6	71
	384	641±6	1.0	82
GC–MS	0	266±3	1.1	
MDA-PH	89	341±6	1.7	84
	178	414±3	0.7	83
	267	505±6	1.2	89

[a] Based on three independent determinations at each fortification level.

spectrophotometric readings (calculated at around 20 μg/kg). However, this limitation does not occur when the spectrum is submitted to the third derivative analysis, which leads to a more selective detection of MDA even if the high-temperature and artefactual formation of MDA is still a problem. The direct reading leads to a 30% overestimation of MDA compared to the results obtained from the third derivative spectrum.

Quantification of MDA-TBA by HPLC gives similar results than those obtained by the third derivative spectrophotometry detection. The relative standard deviations and estimated values observed using these two methods are also similar. These observations underline the fact that the third derivative detection is more specific for malondialdehyde and does not appear to measure ''malondialdehyde-like'' substances. However, quantitation of MDA-TBA measured by HPLC gives values about 2-fold higher than those obtained from the MDA-DNPH analysed by HPLC with UV detection. These discrepancies may be explained by artefactual formation of MDA during the acid heating step required for the TBA derivatisation (70°C), despite the presence of BHT as antioxidant [24,25]. This artefactual formation might be partially skipped by flushing with nitrogen prior to incubate the solution at 70°C for the MDA derivatisation.

Compared to the HPLC–UV detection, the GC–MS technique is far more selective and a 1000-fold more sensitive. Indeed, the limits of detection and quantification of MDA in pure standards were estimated at approximately 1 and 4 pg for the GC–MS versus 1 and 4 ng for the HPLC–UV methods. Moreover, the estimated values calculated from the GC–MS analysis give the most reproducible results confirming this technique as the method of reference to quantify MDA from milk powder samples.

The analytical techniques for MDA detection were further compared using four milk powder samples (A, B, C and D), of similar chemical composition (fortification with PUFA, iron and vitamins C and E) and MDA estimates are summarised in Table 2 and illustrated in Fig. 5. The estimates of MDA by direct visible measurement of MDA-TBA complex are up to 30-fold higher than the values obtained by the

Table 2
Summary of MDA content in four different milk powder samples using various analytical techniques[a] (expressed in μg/kg)

	Visible spectroscopy MDA-TBA (detection at 532 nm)		Third derivative spectroscopy MDA-TBA		HPLC MDA-TBA (detection at 532 nm)		HPLC-MDA DNPH (detection at 307 nm)		GC–MS MDA-PH (SIM Mode)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A	1572	24	767	4	809	7	92	1	51	1
B	1572	33	866	11	848	5	414	4	266	3
C	1046	11	516	5	516	16	99	6	65	0
D	845	40	459	16	385	10	113	2	93	1

[a] Based on three independent experiments for each sample.

GC–MS results. The estimates obtained by HPLC and by the visible method of the MDA-TBA complex after processing the third derivative at 521.5 nm are similar; however, the values overestimate the MDA content calculated by HPLC–UV detection following derivatisation with dinitrophenylhydrazine or by GC–MS. Furthermore, the quantitation of MDA-DNPH derivative analysed by HPLC–UV gives slightly higher values compared to those obtained by GC–MS which may be explained by the absence of internal standard (HPLC method) and the presence of interfering co-eluting peaks.

Based on these results, a classification of MDA content within these four milk powders was done. A similar scale can be observed from the data obtained by the HPLC–UV and GC–MS techniques (A<C<D<B), which is completely different from those coming from the TBA derivatisation. These results indicate that analytical techniques based on TBA derivatisation cannot be used to compare the MDA contents in milk powder samples that do not have identical chemical composition and have not undergone identical processing conditions. However, a different study realised on milk powder samples with identical composition and processing conditions has shown identical proportional results of MDA analysed either by spectrophotometry, UV and GC–MS methods (results not shown). In this case, TBA methods could be used to evaluate the lipid oxidation status [1,26].

4. Conclusion

Quantitation of MDA from milk powder samples using three variants of the TBA test (direct visible,

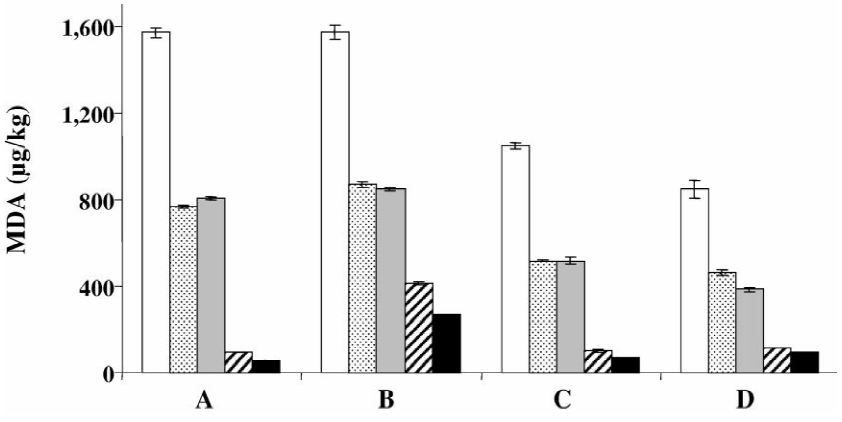

Fig. 5. Comparison of MDA content calculated in four milk powder samples (A, B, C and D) using various analytical methods; (blank area) visible spectrophotometry of MDA-TBA, (dotted area) third derivative spectrophotometry of MDA-TBA, (grey area) HPLC visible of MDA-TBA, (hatched area) HPLC–UV of MDA-DNPH and (black area) GC–MS of MDA-PH.

third derivative and HPLC-visible measurements) appear to overestimate the MDA content because of the high-temperature used during the derivatisation step, which may have catalysed artefactual oxidation of MDA or the formation of other components that interfere with the spectrophotometric assay. The estimates of MDA, determined by both HPLC–UV (DNPH derivative) and GC–MS (PH derivative) techniques lead to lower values, are similar in magnitude even though the GC–MS method is more sensitive than HPLC with UV detection. Moreover, our data suggest that the various TBA assays are much more dependent on the composition of the lipid system than the other techniques investigated.

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References

[1] J.I. Gray, F.J. Monahan, Trends Food Sci. Technol. 3 (1992) 315.
[2] H. Stapelfeldt, G. Mortensen, L.H. Skibsted, Milchwissenschaft 52 (1997) 266.
[3] H. Esterbauer, Am. J. Clin. Nutr. 57 (1993) 779S.
[4] D.R. Janero, Free Radic. Biol. Med. 9 (1990) 515.
[5] J.I. Gray, J. Am. Oil Chem. Soc. 55 (1978) 539.
[6] E.N. Frankel, W.E. Neff, Biochim. Biophys. Acta 754 (1983) 264.
[7] S.L. Melton, Food Technol. 37 (1983) 105.
[8] A.J. Angulo, J.M. Romera, M. Ramirez, A. Gil, J. Am. Oil Chem. Soc. 75 (1998) 1603.
[9] J. Liu, H.C. Yeo, S.J. Doniger, B.N. Ames, Anal. Biochem. 245 (1997) 161.
[10] N.A. Botsoglou, D.J. Fletouris, G.E. Papageorgiou, V.N. Vassilopoulos, A.J. Mantis, A.G. Trakatellis, J. Agric. Food Chem. 42 (1994) 1931.
[11] A. Grau, F. Guardiola, J. Boatella, A. Barroeta, R. Codony, J. Agric. Food Chem. 48 (2000) 1155.
[12] G. Lepage, G. Munoz, J. Champagne, C.C. Roy, Anal. Biochem. 197 (1991) 277.
[13] V.L. Tatum, C. Changchit, C.K. Chow, Lipids 25 (1990) 226.
[14] J. Suttnar, J. Cermak, J.E. Dyr, Anal. Biochem. 249 (1997) 20.
[15] F. Fenaille, P. Mottier, R.J. Turesky, T. Keller, P.A. Guy, in: Proceedings of the Int. Mass Spectrom. Conf., Barcelona, Spain, 2000.
[16] G.A. Cordis, D.K. Das, W. Riedel, J. Chromatogr. A 798 (1998) 117.
[17] N. Deighton, W.J. Magill, D.H. Bremner, E.E. Benson, Free Radic. Res. 27 (1997) 255.
[18] M.A. Shara, P.H. Dickson, D. Bagchi, S.J. Stohs, J. Chromatogr. 576 (1992) 221.
[19] A.W. Bull, L.J. Marnett, Anal. Biochem. 149 (1985) 284.
[20] A.S. Csallany, M. Der Guan, J.D. Manwaring, P.B. Addis, Anal. Biochem. 142 (1984) 277.
[21] W.A. Behrens, R. Madere, Lipids 26 (1991) 232.
[22] E.N. Frankel, in: Lipid Oxidation, The Oily Press, Dundee, Scotland, 1998.
[23] A.M. Salih, D.M. Smith, J.F. Price, L.E. Dawson, Poult. Sci. 66 (1987) 1483.
[24] J.M. Gutteridge, B. Halliwell, Trends. Biochem. Sci. 15 (1990) 129.
[25] B. Halliwell, J.M. Gutteridge, Methods Enzymol. 186 (1990) 1.
[26] D.D. Ward, Milchwissenschaft 40 (1985) 583.