Title: Characterization of the lipid fraction and the water-soluble
metabolites in formula milk

Abstract: In this work, the lipid and water-soluble metabolite profiles
of commercial samples of formula milk (FM) were characterized by
complementary analytical techniques. Analysis of 1H and 13C Nuclear
Magnetic Resonance (NMR) spectra indicated that lipids were mainly
triacylglycerols (TAG) with low quantities of diacylglycerols (DAG), and
that approximately 44-50 mol% of saturated fatty acids were esterified in
sn-2 position of TAG. Phytosterols have been identified in all the
samples. As calculated by high-performance liquid chromatography (HPLC),
the most abundant fatty acids (FA) were oleic acid (C18:1 n-9, mean 42-
47%), palmitic acid (C16:0, 21-32%) and linoleic acid (C18:2 n-6, 13-
20%), and low concentrations of long chain-polyunsaturated FA (LC-PUFA).
Main differences in the 1H NMR metabolite profiles were found in the
sugar contents reflecting the addition of oligosaccharides (OS),
maltodextrins, and sucrose, and for the presence of nucleotides,
tryptophan, and tyrosine. Overall results were discussed in the light of
the nutritional reports.

Highlights

- Metabolites and fatty acids profiling of formula milk

- Formula milk contained phytosterols

- Sucrose was detected in formula milk samples

- One formula milk brand had high tyrosine content

- Nucleotide content was different among formula milk brands

Characterization of the lipid fraction and the water-soluble metabolites in formula milk

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14   In this work, the lipid and water-soluble metabolite profiles of commercial samples of

15   formula milk (FM) were characterized by complementary analytical techniques. Analysis

16   of ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra indicated that lipids were

17   mainly triacylglycerols (TAG) with low quantities of diacylglycerols (DAG), and that

18   approximately 44-50 mol% of saturated fatty acids were esterified in *sn*-2 position of TAG.

19   Phytosterols have been identified in all the samples. As calculated by high-performance

20   liquid chromatography (HPLC), the most abundant fatty acids (FA) were oleic acid (C18:1

21   *n-9*, 42-47%), palmitic acid (C16:0, 21-32%) and linoleic acid (C18:2 *n-6*, 13-20%), and

22   low concentrations of long chain-polyunsaturated FA (LC-PUFA). Main differences in the

23   ^{1}H NMR metabolite profiles were found in the sugar contents reflecting the addition of

24   oligosaccharides (OS), maltodextrins, and sucrose, and for the presence of nucleotides,

25   tryptophan, and tyrosine. Overall results were discussed in the light of the nutritional

26   reports.

27

28   Keywords: infant formula; fatty acids; positional isomerism; tyrosine; tryptophan;

29   nucleotides; phytosterols; sucrose.

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1. Introduction

Human milk (HM) offers the best ~~physiological~~ nourishment to the neonate, with its composition used to ~~estimate~~ the nutritional requirements of infants and to guide the composition of infant formulae. Infant formulae are available on the market in different formulations. Formula milk (FM) ~~is basically~~ prepared by adding to cow milk derivate vegetable oils, vitamins, minerals, and iron. Other components can be added to mimic HM composition and/or for functional and technological purposes. Since the latter do not always correspond to health benefits (Hernell, 2011), the addition of new compounds ~~have~~ to be rigorously evaluated and their suitability proven for infant feeding. In the United States, infant formulae are regulated by the Code of Federal Regulations (Code of Federal Regulations, 2000a and 2000b) and in the European Union by the Commission Directive (Commission of the European Communities, 2006). Although big efforts have been made to achieve the best infant formulas for neonate's nutrition, recent studies highlighted differences in urine (Dessì et al., 2016) and blood (Prentice, Koulman, Matthews, Acerini, Ong, and Dunger, 2015) composition between formula-fed and breast-fed infants, and, in general, different outcomes are reported for the two feeding practices (Obermajer and Pogačić, 2016; Hernell, 2011).

In HM and in most infant formulas, about 50% of the dietary calories are supplied as fat. Lipids are essential for normal growth and development. In general, in HM more than 98% of fat are triacylglycerols (TAG), composed ~~by~~ saturated and unsaturated long- (C24-C12) and medium- (C10-C8) chain fatty acids (FA). As opposite to milk of ruminants, neither short chain FA (C4-C6) nor FA in *trans* configuration were found in relevant concentrations. In HM, the composition of unsaturated FA varies depending on diet and other factors such as day of milking (Sala-Vila, Castellote, Rodriguez-Palmero, Campoy, and López-Sabater, 2005). In contrast, it has been found that level of palmitic acid (C16:0) remains constant over diet and country of origin, and *sn*-2 is its preferential position ~~in~~ the

glycerol backbone of TAG (Straarup, Lauritzen, Faerk, Høy, and Michaelsen, 2006; Innis, 2011). In order to formulate an infant formula with a lipid composition similar to HM, most of the commercial products are based on a mixture of vegetable oils, supplemented by fish oil and other FA sources.

Milk metabolites are hydrosoluble low molecular weight compounds, such as aminoacids, nucleotides, organic acids, mono-, di-saccharides (mostly lactose), and oligosaccharides (OS). They are energy metabolites and are involved in different metabolic pathways, and have a possible nutritional role. Recently, a number of FM are supplemented with GOS (galacto-OS) and FOS (fructo-OS) to mimic HM, rich in OS, and for their prebiotic properties (Boehm, Stahl, Jelinek, Knol, Miniello, and Moro, 2005). Other compounds, such as inositol, carnitine, taurine, nucleotides, and amino acids, can be added to mimic HM composition and for health benefits. In our previous work, exploring the GC-MS metabolite profiles of FM brands compared to HM, different compositions were found between FM and HM but also among FM brands (Scano, Murgia, Demuru, Consonni, and Caboni, 2016).

The aim of this work was to study the lipid composition and the metabolite profiles of FM samples for term infants marketed in Italy. The FA composition was analyzed by HPLC, while the content of TAG, diacylglycerol (DAG), and the positional isomerism of FA in TAG have been studied by ^{1}H and ^{13}C NMR spectroscopy. The metabolite profiles were analyzed by ^{1}H NMR spectroscopy.

To date, few studies employed ^{1}H NMR in the study of metabolites content in FM. Recent studies of Marincola and Longini (Marincola et al., 2012; Longini et al. 2014) compared the ^{1}H NMR metabolite profiles of HM and FM. Lachenmeier and coworkers (Lachenmeier et al., 2009) demonstrated the suitability of routine ^{1}H NMR analysis for the detection of melamine in FM. Differences in the ^{1}H NMR metabolite profiles of different powder infant formulas sold in China were highlighted by Zhao and colleagues (Zhao,

84   Chen, Feng, Chen and Cai, 2017). In regard to the lipid fraction, to the best of our

85   knowledge, the present is the first NMR analysis reported in literature.

86

87   **2. Materials and methods**

88   *2.1. Samples*

89   Samples of 3 different FM brands (coded: A, B and C) sold in Italy, were acquired in the

90   local markets and specialized retailers. They were powder samples of first infant formula

91   (designed for term neonates of 0-6 months of age), based on cow milk derivate with added

92   vegetable oils. List of ~~same~~ ingredients, as reported ~~in~~ the label of samples, is reported in

93   Table 1.

94   *2.2. Chemicals and reagents*

95   Analytical standards of cholesterol, trilinolein, triolein, α-tocopherol, fatty acid methyl

96   esters, Desferal (deferoxamine mesylate salt), and all solvents used, of the highest

97   available purity, were purchased from Sigma–Aldrich (Milan, Italy). D_2O and $CDCl_3$

98   solvents were bought from Euriso-top (Saint-Aubin, France). Standards of β-D-Gal*p*-

99   (1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* (hereafter 3'-galactosyl-lactose), β-D-Gal*p*-(1→4)-β-D-

100   Gal*p*-(1→4)-D-Glc*p* (hereafter 4' galactosyl-lactose), and β-D-Gal*p*-(1→6)-β-D-Gal*p*-

101   (1→4)-D-Glc*p* (hereafter 6' galactosyl-lactose) were bought from Carbosynth Ltd

102   (Compton, UK). All the chemicals used in this study were of analytical grade.

103   *2.3. Extraction and saponification of lipids*

104   Total lipids were extracted from portions of reconstituted milk infant formula samples

105   (approximately 22 mg in 0.5 mL of water, in triplicate), using 9 mL of the mixture

106      $CHCl_3$:MeOH 2:1 (v/v) (Rosa, Murgia, Putzu, Meli, and Falchi, 2015). After addition of

107   H_2O and centrifugation, the $CHCl_3$ fraction was separated from the $MeOH/H_2O$ mixture,

108   dried down and the residue dissolved in EtOH. Separation of lipid components (total

5

109 cholesterol, α-tocopherol, and FA) was obtained by mild saponification as previously

110 reported (Rosa et al., 2015). The unsaponifiable (total cholesterol and α-tocopherol) and

111 saponifiable (FA) fractions were collected and the solvent evaporated. The dried

112 unsaponifiable and saponifiable residues, dissolved in MeOH and CH_3CN with 0.14%

113 CH_3COOH (v/v), respectively, were injected into the high-performance liquid

114 chromatograph. All solvent evaporation was performed under vacuum.

115 *2.4. Analyses of fatty acids, cholesterol, and α-tocopherol*

116 Analyses of α-tocopherol, cholesterol, and FA were carried out with an Agilent

117 Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped

118 with a diode array detector (DAD) (Agilent Technologies) and an Infinity 1260

119 evaporative light scattering detector (ELSD) (Agilent Technologies). Total cholesterol and

120 α-tocopherol, detected at 203 nm and 292 nm, respectively, were measured with the use of

121 an Inertsil ODS-2 column (Superchrom, Milan, Italy), and MeOH as the mobile phase, at a

122 flow rate of 0.7 mL/min. Analyses of fatty acids (unsaturated were detected at 200 nm,

123 saturated with ELSD) were carried out with a XDB-C_{18} Eclipse (Agilent Technologies)

124 equipped with a Zorbax XDB-C_{18} Eclipse guard column (Agilent Technologies), with a

125 mobile phase of $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, v/v/v), at a flow rate of 2.3 mL/min

126 (Rosa et al., 2015). The temperature of the column was maintained at 37 °C. The

127 identification of lipid components was made using standard compounds and conventional

128 UV spectra. Recording and integration of the chromatogram data was carried out through

129 an Agilent OpenLAB Chromatography data system. Calibration curves of compounds were

130 constructed using standards and were found to be linear (DAD), with correlation

131 coefficients > 0.995.

132 *2.5. NMR experiments*.

6

133 Powder formula samples were accurately weighted and diluted in D_2O according to

134 manufacturer's specifications for reconstruction. One mL of $CDCl_3$ was added to 1 mL of

135 solution and vortexed. The mixture was sonicated for 15 min and centrifuged at 12100 *g*

136 for 10 min. The hydrosoluble phase was separated from the organic phase and 0.70 mL

137 were analyzed by ^{1}H NMR spectroscopy while 0.70 mL of organic phase by ^{1}H and [13]C

138 NMR spectroscopy. ^{1}H NMR spectra were acquired at 11.7 T and at 14.09 T NMR

139 spectrometers, (Bruker Avance II 500, and Bruker Avance DRX600 respectively, Bruker

140 Biospin GmbH Rheinstetten, Karslruhe, Germany), equipped with a 5-mm reverse probe

141 with z-gradient. NMR spectra of aqueous and organic fractions were recorded at 298 K

142 with a spectral width of 7184 Hz over 32K points with 256 and 2048 scans respectively.

143 The residual water signal was suppressed by applying a presaturation scheme with a low

144 power radiofrequency irradiation. ^{13}C NMR spectra were acquired at 9.4 T NMR

145 spectrometer (Bruker DRX 400, Bruker Biospin GmbH Rheinstetten, Karslruhe, Germany)

146 with a spectral width of 20.000 Hz over 65.536 points and 80.000 scans. Assignment of [1]H

147 and ^{13}C NMR signals was performed with the aid of literature reports (Zhao et al., 2017;

148 van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2014; Longini et al., 2014; Scano,

149 Anedda, Melis, Lai, and Roggio, 2011; Alonso-Salces et al., 2010; Lachenmeier et al.,

150 2009; Scano et al., 2008), in-house libraries, and by performing two-dimensional (2D) [1]H-

151 ^{1}H total correlation spectroscopy (TOCSY) and [1]H–[13]C heteronuclear single quantum

152 coherence (HSQC) experiments. For the metabolite fraction, spectra were referenced to

153 trimethylsilyl [2,2,3,3-2 H4] propionate (TSP) external standard at 0.00 ppm for both

154 proton and carbon dimensions, while for the lipid fraction tetramethylsilane (TMS) set at

155 0.00 ppm was used as reference. Spectra were processed and analyzed using TopSpin 3.5

156 software (Bruker BioSpin GmbH, version 3.5, Rheinstetten, Karslruhe, Germany).

157 **3. Results**

158 3.1. The lipid fraction

7

159   *3.1.1. NMR analysis of the lipid fraction.*

160   A representative ^{1}H NMR spectrum of the lipid extract in CDCl$_3$ of FM samples is shown

161   in Fig. 1S, while assigned resonances are reported in Table 1S. As shown in Fig. 1S, the

162   spectrum is dominated by resonances due to the functional groups of FA and glycerol in

163   TAG. ==Expanses== of the ^{1}H NMR spectrum of brand A are reported in Fig. 1A, B, C. Brand

164   A, in accordance to the reported butterfat among ingredients, exhibited low intensity

165   signals of milk fat components butyric acid (C4:0, methyl group at 0.94 ppm in Fig. 1A),

166   and, as shown in Fig. 1B, rumenic acid (C18:2 *cis-9, trans-11*, olefinic protons at 6.28,

167   5.93, and 5.65 ppm), and caproleic acid (C10:1 n-1, vinyl protons at 5.81 and 4.95 ppm)

168   (Scano et al., 2011). The signal at 1.66 ppm in Fig. 1C was assigned to the –CH$_2$ in

169   β–position of arachidonic acid (ARA, C20:4 *n-6*,). At very high field, Fig. 1A, the [1]H

170   NMR spectrum exhibited signals ascribable to β-sitosterol at 0.68, 0.81-0.84, 0.91, and

171   0.93 ppm together with the singlets at 1.01 and 1.02 ppm, these latter assigned to its free

172   and esterified forms, respectively (Alonso-Salces et al., 2010; Scano, Rosa, Locci, Dessi,

173   & Lai, 2009; Verleyen et al., 2002). Moreover, signals of low intensity ascribable to 1,2

174   and 1,3 DAG were present in the spectra, their assignment is reported in Table 1S.

175   Although the experiments required a longer time, ^{13}C NMR analysis were carried out in

176   order to obtain information on the positional isomerism of FA in TAG. This issue covers

177   great importance due to the peculiarity of HM having palmitic acid in *sn*-2 position. To this

178   goal, the most informative ^{13}C NMR spectral region is the carboxylic one (Scano et al.,

179   2011). Expansions of the ^{13}C NMR spectrum of sample of brand C are shown in Fig. S2A,

180   B, and C. As shown in Fig. S2A, in the spectral region between 180-170 ppm, there were

181   two main groups of overla==pped== resonances due to the carboxyl carbon atoms of FA in *sn*-

182   1,3 (centered at 173.22 ppm) and *sn*-2 position (centered at 172.80 ppm) in TAG,

183   respectively, detailed assignments are reported in Table S2. From the analysis of the areas,

184   the following results were obtained: FM brands had 29, 25 and 16 mol% of SFA *sn*-2

8

185　position, for A, B and C, respectively. No signals due to the carboxyl carbon atoms of free

186　FA, expected at approximately 178 ppm (Scano et al., 2011), were detectable in the

187　samples, indicating the absence of relevant hydrolytic processes. Moreover, the olefinic

188　carbon region (134-126 ppm) clearly showed a larger content of oleic and linoleic chains

189　respect to the linolenic chain (Fig. S2B). The glycerol carbon region (72-60 ppm, Fig.

190　S2C) is representative of the glycerol carbons carrying acylated or non-acylated alcoholic

191　groups of the glycerol molecule. In this respect, this region confirmed the dominant

192　presence of TAG respect to the 1,3 and the low 1,2 DAG, that are clearly detectable only in

193　brand C. Useful assignments of the ^{13}C NMR spectra of FM samples are reported in Table

194　S2.

195　*3.1.2. HPLC fatty acid composition.*

196　Quali-quantitative information on the individual FA that compose the lipid classes of

197　formula milk samples was obtained by HPLC analyses and fatty acid composition

198　(expressed as % of total fatty acids, g/100 g) is reported in Table 2. Oleic acid was the

199　most abundant FA with concentrations (mean±SD) of 42.1±0.8, 47.6±1.5, and 47.2±0.8 %

200　for A, B, and C, respectively, followed by palmitic acid (21.1±1.2, 24.2±2.3, and 31.9±0.9

201　% for A, B, and C, respectively). The third most abundant FA was the linoleic acid

202　(20.4±0.7, 16.4±0.6, and 13.4±0.2%, for A, B, and C, respectively). Brands A and B had

203　comparable concentrations of the *n-3* LC-PUFA, with higher amount of docosahexaenoic

204　acid (DHA C22:6 *n-3*, 0.31±0.01 and 0.28±0.02 %, for A and B samples, respectively)

205　over eicosapentaenoic acid (EPA C20:5 *n-3*). The ARA concentrations were 0.50±0.01 and

206　0.23±0.01% for A and B samples, respectively. LC-PUFA were not detected in samples of

207　brand C.

208　*3.1.3. Vitamin E and cholesterol.*

209 The concentrations of vitamin E were 11.94±2.00, 6.31±0.49, and 8.37± 0.81 mg/100 g of

210 milk in samples of A, B, and C brands, respectively. Cholesterol was not detected in FM

211 samples.

212 **3.2. Metabolites**

213 *3.2.1. ^{1}H NMR metabolite profile.*

214 As shown in Fig. S3, the ^{1}H NMR spectra of the aqueous fraction are dominated by the

215 signals of lactose. Expansions of the ^{1}H NMR spectra are shown in Fig. 2A, B, C and D

216 and the assignment of resonances is reported in Table S3. Main differences among brands

217 were detected in the aromatic spectral region (9.80-5.80 ppm, Fig. 2A) and in that related

218 to the presence of sugars (5.60-3.20 ppm, Fig. 2B and C).

219 Brands A and C exhibited the characteristic signal of maltose at 3.41 ppm (Longini et

220 al., 2014) probably as maltodextrin unit (Fig. 2C). In brand B, sucrose was identified by its

221 resonances at 5.44, 4.25, and 4.09 ppm (Zhao et al., 2017; Lachenmeier et al., 2009),

222 together with free glucose, identified by the resonances at 4.63 ppm and in the 3.50-3.36

223 ppm spectral region. Galactose-1-phosphate was detected in all samples (Longini et al.,

224 2014). As shown in Fig. 2B, the broad signal at 4.17 ppm that could refer to the H4 β-

225 galactose GOS, and in particular of the 3'-galactosyl-lactose, the 4'-galactosyl-lactose with

226 three or more monosaccharide units, and of the 6'-galactosyl-lactose with more than 3

227 monosaccharide units, was clearly detectable only in B brand. The analysis of the HSQC

228 spectra of B sample and of the standard compounds together with the aid of literature data

229 (van Leeuwen et al., 2014), let to confirm the presence of both the 3'-galactosyl-lactose

230 and the 4'-galactosyl-lactose with three monosaccharide units and to exclude the presence

231 of 6'- galactosyl-lactose.

232 Among free amino acids, main differences were visible in the aromatic spectral region

233 (Fig. 2A). Here, brand A exhibited a much higher quantity of tyrosine (peaks at 6.89 and

234 7.19 ppm, see expansion in Fig. 4S) while tryptophan was detectable in spectra of brands A

235 and B. Leucine, isoleucine, lysine, alanine, glutamic acid, and asparagine were present in
236 all brands.

237 Protons of nucleobase rings resonate in the aromatic region (Fig. 2A). Here, the
238 nucleotides adenosine-5'-monophosphate (5'-AMP), inosine-5'-monophosphate (5'-IMP)
239 and cytidine were detected in brand B. Adenine, cytidine-5'-monophosphate (5'-CMP),
240 and uridine-5'-monophosphate (5'-UMP) were detectable in brands A and B. Uridine and
241 orotic acid were found in all samples of the three brands. In the region 4.8–4.0 ppm (Fig.
242 2B) resonated protons of the ribose unit of the nucleosides and the 5'-nucleotides.

243 The organic acids: lactate, citrate, and formate were detected, together with acetate the
244 N-acetyl groups, and creatine. Moreover, choline, phosphocholine and
245 glycerophosphocholine, niancin, and ascorbic acid, were identified in all the three brands.
246 Taurine resonances, buried in the region of sugars, were detectable only by 2D
247 experiments.

4. Discussion

249 HM has, generally, a relatively high content of oleic acid (30-40%) and palmitic acid
250 (20-30%) (Innis, 2011; Straarup et al., 2006; Sala-Vila, 2005). These values were roughly
251 matched by the three analyzed FM brands, which reported vegetable oils as fat ingredient
252 (Table 1). Palm oil was the first listed oil for brands B and C. Palm oil is a tropical oil with
253 a well-balanced amount of palmitic acid (also in *sn*-2 position in TAG) and oleic acid
254 (Jurriens, De Vries, and Schouten, 1964). Brand A had a declared content of sunflowers oil
255 as vegetable oil, which is highly unsaturated with a high content of linoleic acid, in
256 agreement the concentration % of linoleic acid (20.42±0.75) was higher in this brand,
257 when compared with the others (16.45±0.56 and 13.36±0.21, for B and C samples,
258 respectively) and with the literature data for HM (13-8%, Straarup et al., 2006). Rapeseed
259 oil was present in all the brands. Diverse typologies of this oil, naturally enriched in the

erucic acid (C22:1 *cis*-13) that has adverse effects on human health, are now present on the market with a reduced erucic acid content.

HM contains more than half of the palmitic acid content esterified in the *sn*-2 position in the glycerol backbone of TAG. The positional distribution of palmitic acid is significant because, in the intestinal tract, pancreatic lipase digests the TAG to form two free FA, releasing the FA in *sn*-1,3 positions and a 2-monoacylglycerol (2-MAG) (Innis, 2011). The long chain saturated fatty acids, e.g. palmitic acid and stearic acid, esterified in the *sn*-1,3 positions, after digestion are released as free fatty acids and relatively poorly absorbed, thus forming insoluble soap with calcium; this is in contrast to the better absorption of these acids as 2-MAG (Innis, 2011; Straarup et al., 2006). Brands B and C, unlike brand A, contained palm oil. Palm oil is unique among vegetable oils in having a significant amount of palmitic acid (44%) which occupies approximately 11% of the *sn*-2 position in TAG (Jurriens et al., 1964). The presence of palmitic acid in brand A ~~can be~~ due to the added butter fat where it occupies the *sn*-2 position in two-thirds of TAG species (Karupaiah, and Sundram, 2007). As reported in Table 2, among SFA, palmitic acid was by far the most concentrated FA in the three brands, therefore, it is possible to assume that in FM samples the SFA that occupied the *sn*-2 position in TAG was mostly palmitic acid.

HM contains also n-3 LC-PUFA, mainly DHA and EPA, in low (< 0.5%) and very variable concentrations (Innis 2011; Straarup et al., 2006; Sala-Vila, 2005). For a normal infant growth, enrichment of FM with LC-PUFA is suggested (Guo and Ahmad, 2014). In the three brands the presence of *n-3* LC-PUFA was guarantee by the addition of fish oil, a natural source of DHA and EPA (Ward and Singh, 2005). The addition of LC-PUFA in infant formulae for term infants, with appropriate regard for quantitative and qualitative qualities, is safe and enables the formula-fed infant to achieve the same blood LC-PUFA *status* as that of the breast-fed infant (Fleith and Clandinin, 2005). The examined formula milk samples had DHA at 0.31 and 0.28 % for brands A and B, respectively, values

286   slightly lower than the required intake set at 0.4% (Guo and Ahmad, 2014). The content of

287   the ARA, an *n-6* LC-PUFA, was relevant only in samples of brand A (0.50±0.05 %) and in

288   a lower content in brand B. ARA was one of the ingredients in brand A. To provide an

289   intake of ARA, brand B listed oil from *Mortierella alpine* among its vegetable oil

290   ingredients. *Mortierella alpina* is an oleaginous fungus capable to accumulate large

291   quantity of oil rich in ARA (Ward and Singh, 2005). No LC-PUFA were detectable in

292   brand C. During neonatal life, there is a rapid deposition of ARA and DHA in an infant's

293   brain, of DHA in retina, and ARA in the whole body (Guo and Ahmad, 2014). From 1990

294   onwards, a number of health and nutrition organizations specifically recommended

295   inclusion of ARA and DHA in pre-term and term infant formula. Some authors, due to

296   their role in infant growth, suggest that the presence of DHA should be balanced by a

297   proper amount of ARA, with a ratio ARA:DHA of approximately 1.5 (Guo and Ahmad,

298   2014), this in contrast with European Food Safety and Authority (ESFA) recommendation

299   where ARA is not required "even in the presence of DHA" (EFSA, 2014). Samples of A

300   and B brands had the ARA:DHA ratio of 1.6 and 0.8, respectively, the former in good

301   agreement with the recommended value (Guo and Ahmad, 2014) .

302   In agreement with the addition of butterfat as fatty ingredient, the presence of rumenic

303   acid was detected in samples of brand A. Although the health beneficial role of this FA is

304   amply studied, to the best of our knowledge, no data on the role of rumenic acid in

305   neonatal nutrition are reported in the scientific literature.

306   HM contains cholesterol (Hendricks and Guo, 2014). Cholesterol is found in foods of

307   animal origin, including milk fat. Cholesterol is synthesized in the human organism, and

308   this capacity for synthesis seems to be well developed at birth (Guo and Ahmad, 2014).

309   Cholesterol is needed by the infant in challenging the development of cholesterol

310   metabolizing enzymes and it contributes to synthesis of nerve tissue and bile salts.

311   Generally, cholesterol was found very low in FM compared to HM (Koletzko, Rodriguez-

312   Palmero, Demmelmair, Fidler, Jensen, and Sauerwald, 2001; Cruz et al., 1994). In the here

313   examined samples, cholesterol was not detectable neither by HPLC analysis nor in the

314   NMR spectra. Vegetable oils contain small amounts of plant sterols in their free and

315   esterified forms (Verleyen et al., 2002), mainly β-sitosterol, stigmasterol, and campesterol.

316   β-sitosterol was detectable in the ^{1}H NMR spectra of FM samples. It has been reported that

317   plant sterols are poorly absorbed (5–15%) by the intestine but interfere with the absorption

318   of cholesterol (Guo and Ahmad, 2014), therefore, in nutritional studies, the presence of

319   phytosterols in FM has to be taken into consideration.

320   The higher content of vitamin E in brand A is consistent with the addition of extra

321   virgin olive oil (see Table 1), rich in this liposoluble vitamin.

322   The NMR analysis reported the presence of DAG particularly in the lipid fraction of

323   brand C. No information regarding their addition to FM samples was reported in the list of

324   ingredients. Structured lipids can be obtained by the appropriate acylation of desired

325   position in glycerol, for example palmitic acid (or DHA) can be esterified in the desired *sn*-

326   2 of the glycerol backbone. In order to supply an amount of palmitic acid in *sn*-2 position,

327   some formulations implement the lipid fraction with a blend of DAG and MAG having

328   palmitic acid in *sn*-2 position. However, a small quantity of DAG is also expected in

329   refined vegetable oils. Acyl migration is observable in the shift of an acyl-group from *sn*-2

330   position of 1(3),2 DAG to the *sn*-1(3) position, so to form the more stable 1,3 DAG

331   (Laszlo, Compton, and Vermillion, 2008). Consistently, higher quantities of 1,3 DAG were

332   found in our samples.

333   In FM, the low molecular weight compounds can be either present in the fractions of

334   cow milk used or added to furnish the right amount of necessary nutrients for normal

335   growth, as suggested by the international committees (Thompkinson, and Kharb, 2007). In

336   HM, among sugars, lactose is the main source of energy. Lactose was supplement in the

337   three formulations. Besides, A and B brands reported the addition of GOS and FOS.

14

Prebiotic OS are non-digestible saccharides that pass the gastrointestinal tract intact and are selectively fermented by the gut flora in the colon (Boehm et al., 2005). There is evidence that OS in HM (HMO) are important for their prebiotic effect (essentially bifidogenic) as well as the antiinfective and allergy-preventive properties of HM. One characteristic of HMO is the large amount of galactose, the backbone structure is based on lactose plus a further galactose residue forming the different galactosyl-lactoses, mainly 3'-galactosyl-lactose, 4'-galactosyl-lactose and 6'-galactosyl-lactose. Larger OS are formed by repeated units of galactose-N-acetylglucosamine added to the core lactose. The backbone is further modified by the specific addition of fucose and sialic acid residues (Hendricks and Guo, 2014). The Commission Directive 2006/141/EC states "Fructo-oligosaccharides and galacto-oligosaccharides may be added to infant formulae. In that case their content shall not exceed: 0.8 g/100 ml in a combination of 90% oligogalactosyl-lactose and 10% high molecular weight oligofructosyl-saccharose". This mixture was designed also to mimic the molecular size distribution found in the neutral fraction of HMO (Boehm et al., 2005). GOS can be obtained from lactose hydrolysis by β-galactosidase, and it has been reported that GOS obtained by this procedure can contain large amounts of glucose, galactose, and unreacted lactose, which do not have prebiotic properties and increase the calorific value of the product (Cardelle-Cobas, Corzo, Villamiel, and Olano, 2008). FOS (e.g. fructo-oligosaccharides, oligofructose, inulin) are composed of glucose and repetitive fructosyl residues in β-2-1 linkage or β-2-6 linkage. FOS are usually extracted from the root of chicory and further enzymatically digested to oligofructose. An alternative is the enzymatic synthesis of oligofructose from sucrose (Boehm et al., 2005). Analysis of 1D and 2D NMR spectra allowed detection of 3'-galactosyl-lactose and 4'-galactosyl-lactose in B brand.

Sucrose and fructose are sweeter than lactose, glucose, maltose, and glucose polymers. Because of sweetness, infants fed *ad libitum* tend to consume more volume of a formula

containing sucrose compared to lactose. The addition of sucrose, unless needed, should be avoided in infant formula, also because of potential life-threatening symptoms in young infants with unrecognized hereditary fructose intolerance (Nguyen, Bhandari, Cichero, and Prakash, 2015). However, sucrose may be helpful in camouflaging the bitter taste of protein hydrolysates, therefore its use is allowed in formulates based on protein hydrolysates in amounts of up to 20% of the total carbohydrate content (Nguyen et al., 2015). Sucrose was detected in samples of brand B, this brand enlisted L-tryptophan among the ingredients and having this amino acid a bitter taste (Solms, 1969) sucrose can help in camouflaging it. The FM concentration of galactose was found, on average, higher than in HM (Scano et al., 2016; Cavalli, Teng, Battaglia, and Bevilacqua, 2006). Galactose is an important carbohydrate for energy production in neonates being the main substrate for hepatic glycogen synthesis (Kliegman and Sparks, 1985). Longini et al. (2014) reported a higher level of galactose-1-phosphate in FM in comparison to HM.

According with labels, maltodextrins were found in brand A and C. Maltodextrins are thickening compounds that render powdered milk more soluble, they are less sweet than glucose and release energy slower. Because of the chain-length specificity of intestinal glucoamylase, in FM maltodextrins with 5 to 9 glucose units should be preferred (Tompkinson et al., 2007; Nguyen et al., 2015). At present, the type of glucose polymers is not regulated, this is reflected in the variable pattern of distribution of the degree of polymerization in commercial formulae containing maltodextrin, which ranged from 1 to 30 units of glucose (Nguyen et al., 2015).

In FM, a balanced content of amino acids is mandatory (Thompkinson et al, 2007). Amino acids in their free form are more easily adsorbed than those present in proteins and may have a beneficial role during early post-natal development (Agostoni, Carratù, Boniglia, Riva, and Sanzini, 2000). The Commission of the European Communities states: "amino acids may be added to infant formulae solely for the purpose of improving the

nutritional value of the proteins, and only in the proportions necessary for that purpose" (Commission of the European Communities, 2006). In this regard, brands A and B reported tryptophan, and brand B tyrosine in their ingredient list. In the neonate, tryptophan and its metabolites are essential for optimal cerebral development, including the correct development of the hunger, satiety and sleep-wake rhythm regulation systems (Heine, 1999). HM contains elevated concentrations of tryptophan, the precursor of serotonin and melatonin, compared with other neutral amino acids, and tryptophan transport across the blood–brain barrier is optimal (Heine, 1999). In contrast, cows' milk formula provides lower tryptophan levels with higher concentrations of neutral amino acids therefore, it has been suggested to supplement FM with tryptophan to a level similar to what is present in HM (Heine, 1999). In accordance with the ingredient list, tryptophan has been detected in FM brand B and in lower quantity in brand A. Moreover, brand A showed a relatively high concentration of tyrosine. A tyrosine content higher than HM has been already reported for some FM brands (Zhao et al., 2017; Scano et al., 2016; Agostoni et al., 2000), health implications of this issue should be evaluated. Moreover, L-tyrosine has a bitter taste (Solms, 1969) and its impact in FM flavor has to be considered.

Brand B added the nucleotides 5'-CMP, 5'-UMP, 5'-AMP, 5'-GMP, and 5'-IMP, while brand A enlisted nucleotides among the ingredients without further details. Nucleotides and their metabolic products are present in human and animal milk (Hendricks and Guo, 2014). Nucleotides play key roles in many biological processes. Studies of infants fed formula fortified with nucleotides at concentrations equivalent to the free nucleotide concentration of HM (10–29 mg/L) have reported beneficial effects on immune system and a decreased incidence of diarrhea (Gutiérrez-Castrellón et al., 2007). Currently, the addition of 5'-CMP, 5'-UMP, 5'-AMP, 5'-GMP, 5'-IMP to infant formulas is allowed but not mandatory (Commission of the European Communities, 2006). In agreement to the label statement, the presence of 5'-CMP, 5'-UMP, 5'-AMP, and 5'-IMP was detected in

416   brand B. 5'-CMP, 5'-UMP were detectable also in brand A, while brand C contained only

417   uridine, probably from the cow milk used (Gill, Indyk, and Manley-Harris, 2011). Orotic

418   acid, a pyrimidine, probably from cow milk, was detected in all brands. Differently from

419   cow milk, HM do not contain orotic acid in appreciable quantities (Scano et al., 2016). The

420   effect on neonates' health of orotic acid is matter of debates (Karatas, 2002).

421   Among low molecular weight compounds, according with labels, niancin, choline,

422   taurine, and ascorbic acid were also detected in the three brands.

423   **5. Conclusions**

424   Results of this work indicate that FM brands had some differences in their composition

425   mainly due to the different ingredients used. Considering that these compositional

426   differences could have different impact in neonate's health, it is our opinion that nutritional

427   researches on formula-fed infants would benefit of a deeper knowledge on the composition

428   of the specific infant formulations used. Moreover, manufacturing technologies and the

429   nutrient extractive sources have to be carefully monitored to avoid the presence of by-

430   products and/or of hidden compounds.

431

432   **Conflict of interest**

433   The authors declare no conflict of interest.

434

435   **Ethical statement**

436   This article does not contain any studies with human participants or animals performed

437   by any of the authors.

438

439   **Informed consent**

440   Not applicable.

441

18

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581

Caption of figures.

Fig. 1. Expansions of the 600 MHz ^{1}H NMR spectrum of the lipid extract in CDCl$_3$ of brand A. NMR signals were assigned as reported in Table 1S. A) 0.60-1.04 ppm: –CH$_3$ functional groups of 1) β-sitosterol H18, 2) β-sitosterol, 3) butyric acid, 4) *n-3* PUFA, 5) free β-sitosterol H19, 6) esterified β-sitosterol H19, S) ^{13}C satellites.; B) 1.58-2.90 ppm: 1) –CH$_2$- in β position of FA; 2) –CH$_2$- in β position of ARA; 3) allylic –CH$_2$ in MUFA; 4) allylic –CH$_2$ in PUFA; 5) –CH$_2$- in α-position of FA; 6) *bis*-allylic –CH$_2$ in linoleic acid; 7) *bis*-allylic –CH$_2$ in PUFA. C) 4.80-6.40 ppm, olefinic groups of 1) caproleic acid H9; 2) rumenic acid H12; 3) caproleic acid H10; 4) rumenic acid H10; 5) rumenic acid H11, and 7) CH- of glycerol in 1,2 DAG.

Fig. 2. Expansions of the 600 MHz ^{1}H NMR spectra of aqueous extract in D$_2$O for FM samples of brand A, B, and C. NMR signals were assigned as reported in Table 3SM. A) 9.00-5.80 ppm: 1) niancin, 2) formate, 3) 5'-AMP, 4) 5'-IMP, 6) 5'-CMP and 5'-UMP, 7) uridine, 8) hippurate, 9) tryptophan, 10) tyrosine, 11) orotate, B) 5.50-4.10 ppm: 12) sucrose, 13) galactose-1P, 14) galactose, 15) glucose, 16) ascorbate, 17) GOS, 18) choline, C) 3.50-3.15 ppm: 19) maltose, 20) phosphocholine and glycerophosphocholine; D) 3.40-0.98 ppm: 21) creatine, 22) lysine, 23) citrate, 24) glutamate, 25) N-acetyl groups, 26) acetate, 27) lactate, 28) leucine and isoleucine.

Table1

Table 1. Macronutrients and some micronutrients[a], as reported in the ingredient list[b], for FM brands.

| Brand	Protein source	Fat source	Carbohydrate source	Prebiotics[c]	Micronutrients
A	Milk whey, skimmed milk, whey protein	Vegetable oils (sunflowers oil, rapeseed oil, extra virgin olive oil), ARA, refined fish oil, soy lecithin, butter cream.	Lactose, maltodextrins	GOS, FOS	Tyrosine, inositol, taurine, nucleotides, tryptophan, carnitine, niacin
B	Milk whey, skimmed milk, whey proteins	Vegetable oils (palm oil, low erucic rapeseed oil, coconut oil, sunflowers oil, *Mortierella alpina* oil), fish oil, soy lecithin.	Lactose	GOS, FOS	Choline, taurine, nucleotides[d], tryptophan, inositol, niacin, carnitine
C	Skimmed milk, whey proteins	Vegetable oils (palm oil, rapeseed oil, palm kernel oil, sunflowers oil), fish oil, soy lecithin.	Lactose, maltodextrins		Niacin, choline, taurine

a) other ingredients, such as mineral salts and vitamins, were reported in the ingredients list; b) nutrients are reported in the same order of the ingredient list; c) galactooligosaccharides (GOS) and fructooligosaccharides (FOS); d) 5'-CMP, 5'-UMP, 5'-AMP, 5'-GMP, 5'-IMP.

Table2

Table 2. Fatty acid composition (% of total fatty acids) by HPLC of FM samples.

Fatty acids	Brands		
	A	B	C
C14:0	2.24 ± 0.19^a	3.43 ± 0.18	1.43 ± 0.11
C14:1	0.52 ± 0.01	-	-
C16:0	21.05 ± 1.23	24.25 ± 2.31	31.87 ± 0.89
C16:1 *n-7*	0.92 ± 0.02	0.23 ± 0.05	0.22 ± 0.06
C18:0	8.27 ± 0.38	3.71 ± 0.36	3.52 ± 0.18
C18:1 *n-9*	42.06 ± 0.76	47.62 ± 1.54	47.15 ± 0.77
C18:1 *trans*	1.02 ± 0.04	-	-
C18:2 *n-6*	20.42 ± 0.75	16.45 ± 0.56	13.36 ± 0.21
C18:3 *n-3*	2.50 ± 0.04	3.57 ± 0.09	2.35 ± 0.03
C18:3 *n-6*	0.10 ± 0.01	0.17 ± 0.01	$0.09+ \pm 0.01$
C20:4 *n-6*	0.50 ± 0.01	0.23 ± 0.01	-
C20:5 *n-3*	0.08 ± 0.00	0.06 ± 0.00	-
C22:6 *n-3*	0.31 ± 0.01	0.28 ± 0.02	-
SFA[b]	31.56 ± 1.55	31.39 ± 2.23	36.82 ± 1.03
MUFA[c]	44.52 ± 0.77	47.85 ± 1.55	47.38 ± 0.79
PUFA[d]	23.91 ± 0.79	20.75 ± 0.68	15.80 ± 0.24

a) Mean and standard deviation over 4 samples; b) SFA, saturated fatty acids; c) MUFA, monounsaturated fatty acids; d) PUFA, polyunsaturated fatty acids.
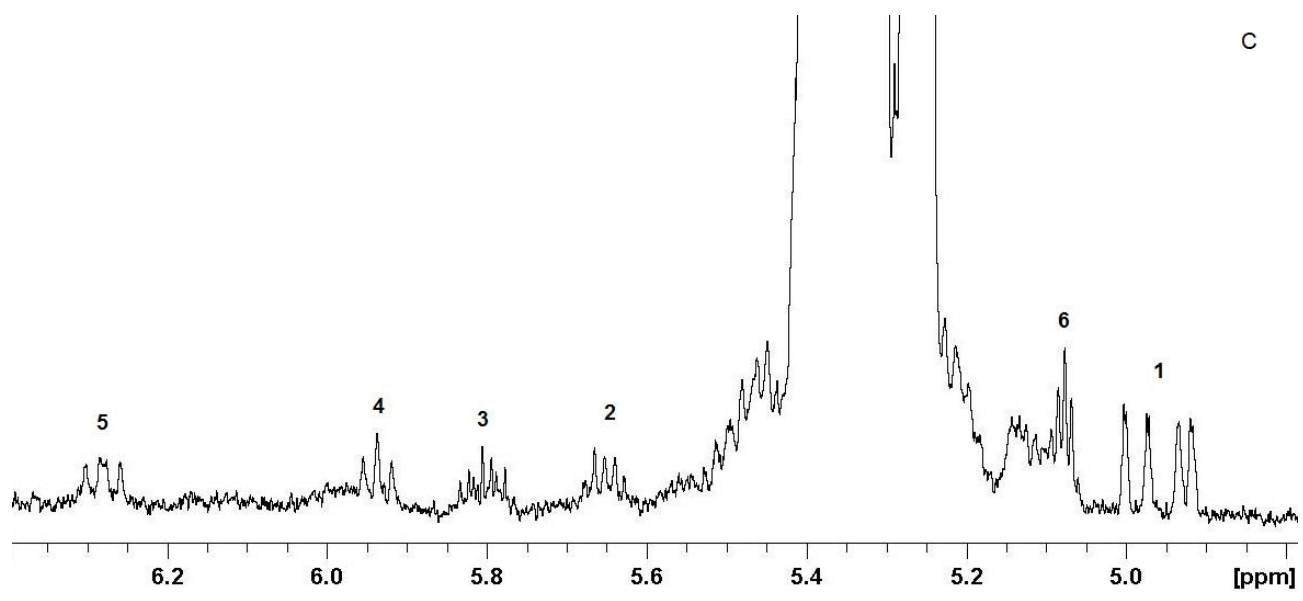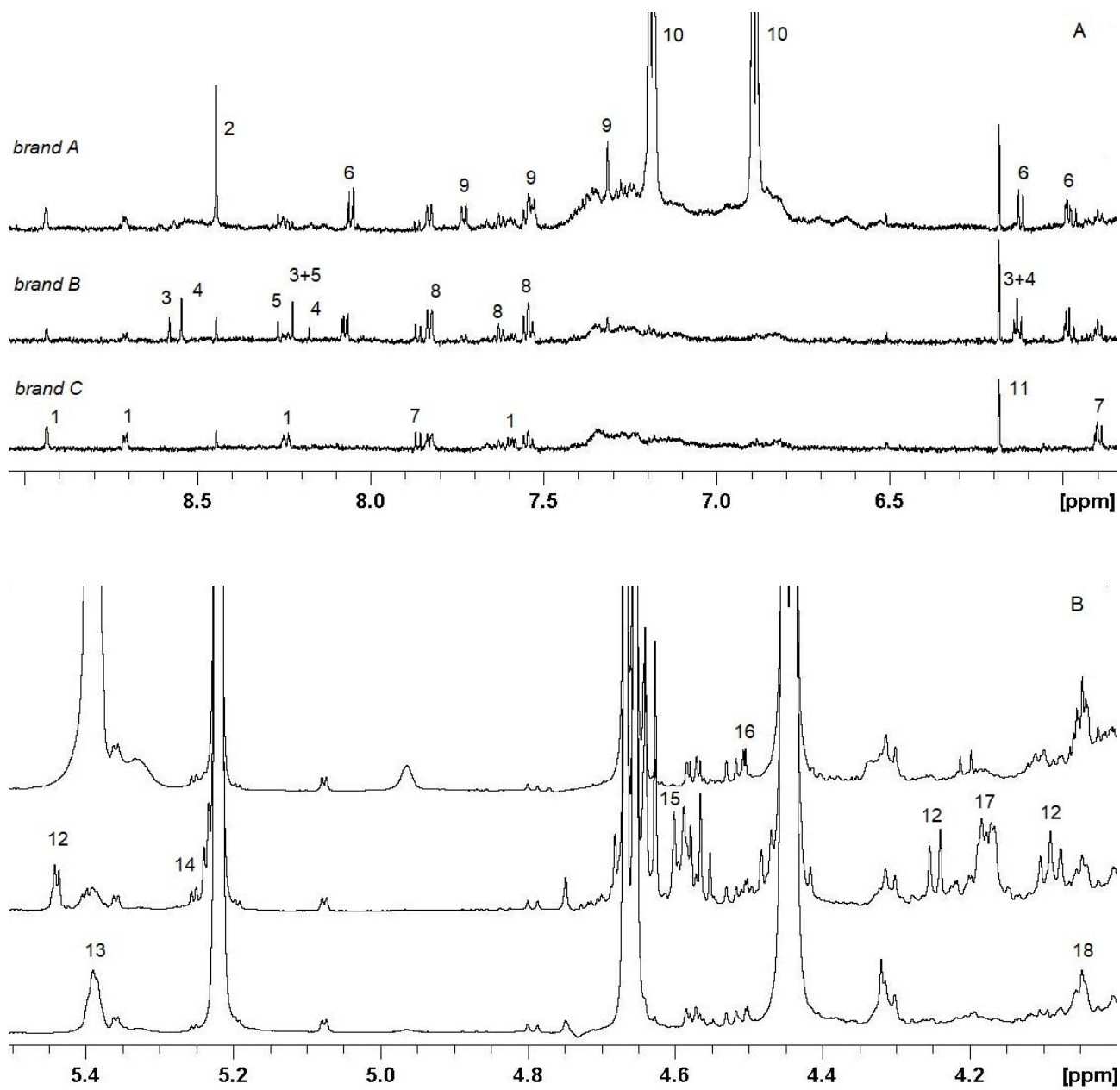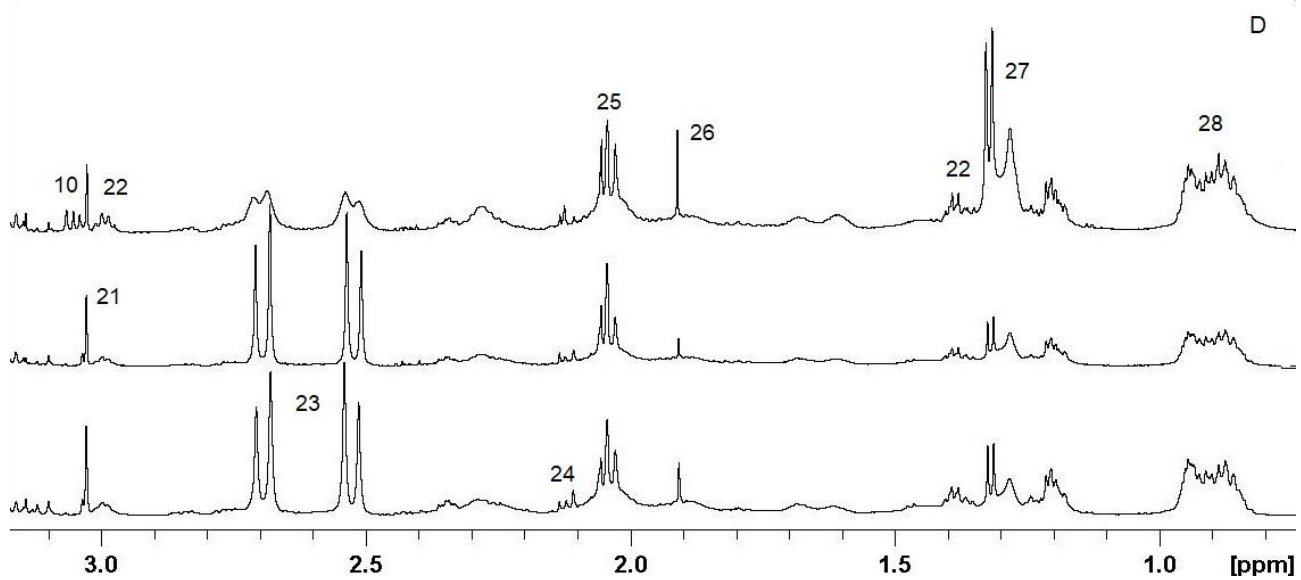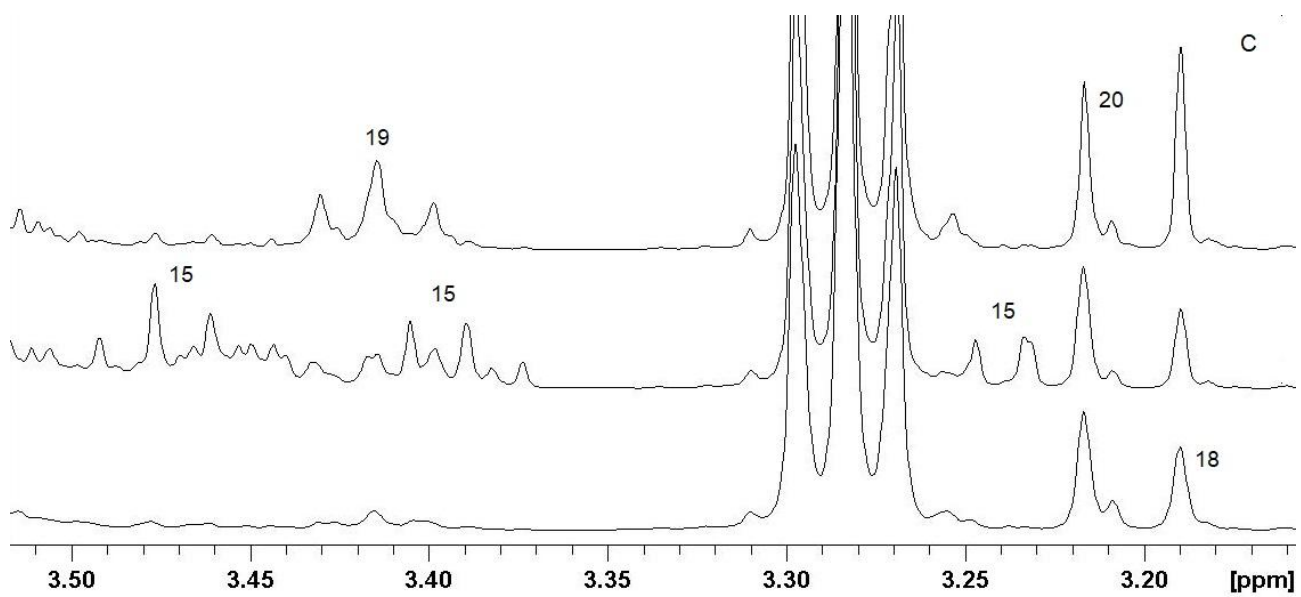
Fig.1
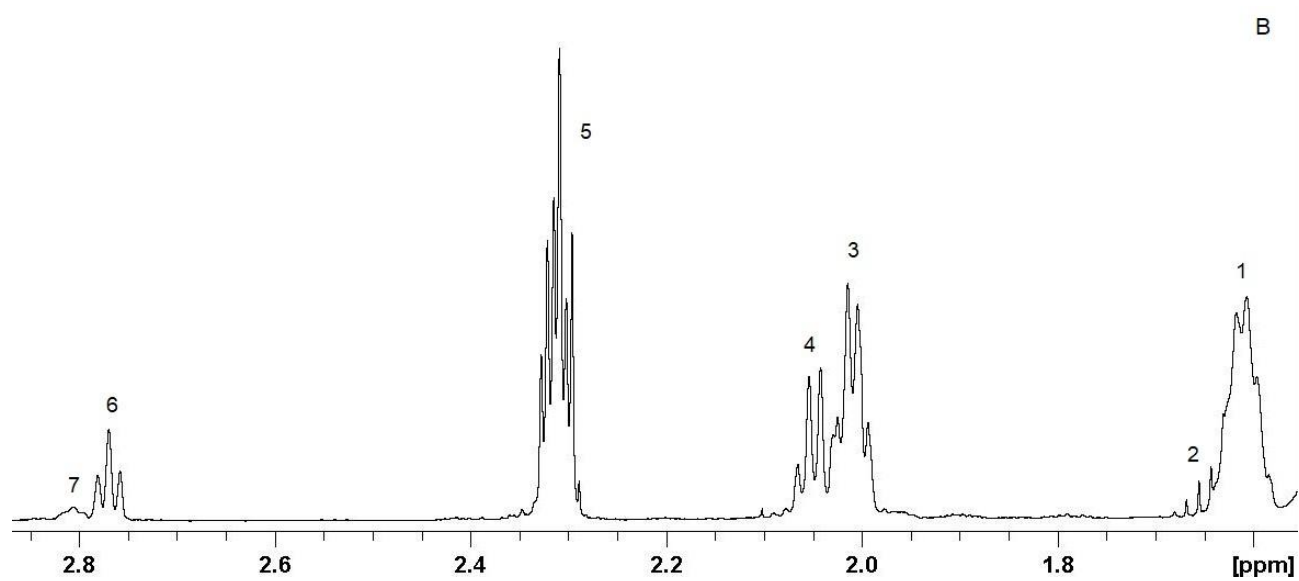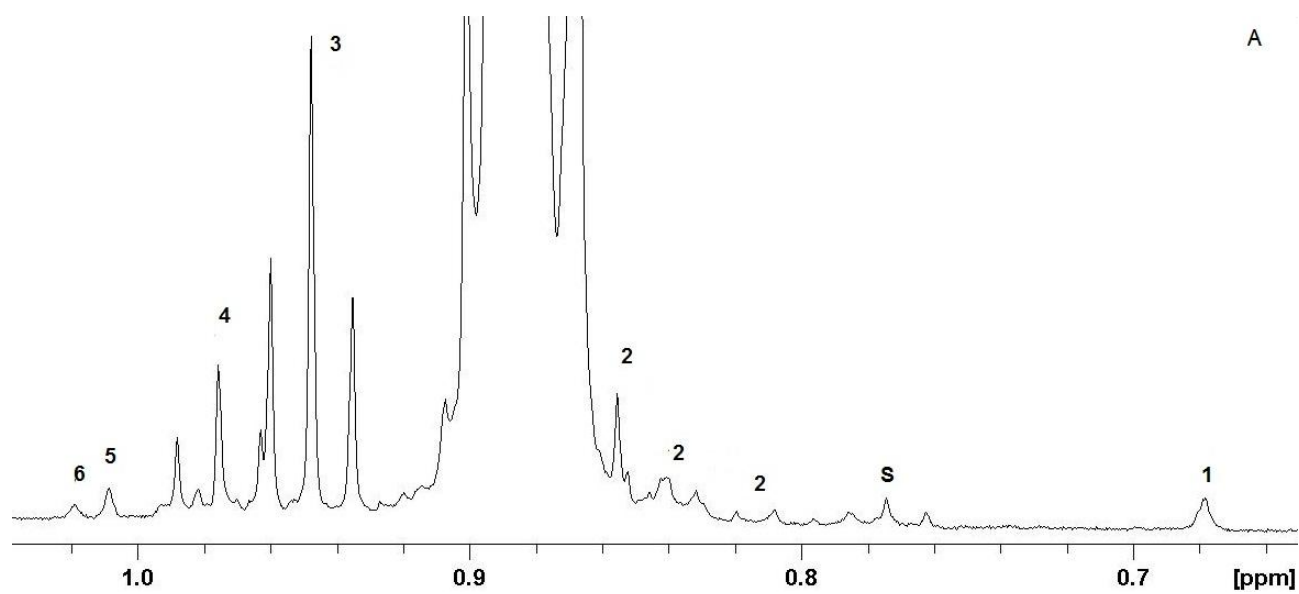
Fig.2

Supplementary Material
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