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Concentrations of phytoestrogens in conventional, organic and free-range retail milk in England

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Highlights

- Phytoestrogens in milk from three management systems were measured
- Organic milk contained more lignans, isoflavones and coumestants
- Phytoestrogen composition did not vary between free-range and conventional milk
- Summer milk contained more lignans and less isoflavones than winter milk
- Milk from different production systems may alter consumer phytoestrogen intake

Abstract

The effect of dairy management system (conventional, CNV; organic, ORG; free-range, FRG) and month on retail milk phytoestrogen composition was assessed for 12 consecutive months. ORG milk contained more secoisolariciresinol, matairesinol, lariciresinol, sum of plant lignans, daidzein, genistein, formononetin, naringenin, equol, sum of isoflavones and coumestrol, than CNV and FRG milk. This may be explained by the higher supply of pasture, and grazed or ensiled clover, in ORG dairy diets. Seasonal variation in milk phytoestrogen concentrations was higher for ORG than CNV and FRG systems. Phytoestrogen composition did not vary between FRG and CNV milk. Consuming organic milk can increase intake of potentially beneficial lignans and isoflavonoids, and in particular equol; but, any effects on human health from such milk compositional differences cannot be implied.

1. Introduction

Milk is an important part of healthy and balanced diets, because it contains high biological value proteins, bioactive peptides, fatty acids, minerals, vitamins, and carotenoids, with multiple benefits in human health (Thorning, Raben, Tholstrup, Soedamah-Muthu, Givens, & Astrup, 2016). Previous work has shown that milk also contains phytoestrogens, such as isoflavones, lignans and coumestans (Kuhnle, Delcaquilla, Aspinall, Runswick, Mulligan, & Bingham, 2008), of which the potential effect in human health is not extensively investigated and nutritional recommendations are not available (Leitzmann, 2016). Phytoestrogens are plant secondary metabolites and involved in plant development and survival (Crozier, 2009). Lignans are bound to cell wall macromolecules, because they are formed during lignin synthesis and strengthening of the plant cell wall (Kuhnle, Dell'Aquila, Aspinall, Runswick, Mulligan, & Bingham, 2009b). Linseed and grains, especially wheat and rye, are rich in lignans, which are located in the aleurone layer of the bran (Fardet, 2010; Kuhnle, et al., 2009b; Smeds, et al., 2007). In lower amounts, lignans also exist in fruits, vegetables, grasses and legumes (Adler, Purup, Hansen-Moller, Thuen, Gustavsson, & Steinshamn, 2014; Kuhnle, et al., 2009a). Isoflavones are produced by *Fabaceae Leguminosae* plants, and perform various functions, providing mainly defense against pathogens (Adler, et al., 2014). Soybeans are the richest source of the isoflavones daidzein and genistein, whereas red clover is rich in formononetin and biochanin A, but has low daidzein and genistein concentrations (Mustonen, et al., 2009). White clover contains less total isoflavones than red clover, but more lignans and coumestans (Adler, et al., 2014; Andersen, Weisbjerg, Hansen-Moller, & Sejrsen, 2009; Hojer, et al., 2012; Mustonen, et al., 2009; Steinshamn, Purup, Thuen, & Hansen-Moller, 2008). The concentration of coumestans in plants increases in response to stress or diseases (Reed, 2016) and coumestrol has been found in 58 plants, being in high amounts in legumes, such as white clover, lucerne and peas (Reed, 2016).

26 In cows, phytoestrogens are mostly metabolized in the rumen, and therefore the transfer
27 rates from feed to milk are small (Gagnon, et al., 2009; Njastad, Adler, Hansen-Moller, Thuen,
28 Gustavsson, & Steinshamn, 2014). The rumen metabolism of lignans, isoflavones and
29 coumestans is complex and their degree of conversion varies among different phytoestrogens
30 (Adler, et al., 2014; Heinonen, et al., 2001; Njastad, et al., 2014). Njastad et al. (2014) showed
31 that plant isoflavones were extensively metabolized in the rumen (70% and 90% of biochanin
32 A and genistein, respectively) into intermediary compounds. Most of the formononetin and
33 daidzein was also transformed in the rumen into the mammalian isoflavone equol (Njastad, et
34 al., 2014). Rumen microorganisms also extensively metabolise plant lignans into the
35 mammalian lignans enterodiol and enterolactone (Heinonen, et al., 2001; Njastad, et al., 2014),
36 while animal studies and experiments with human fecal inoculum showed that
37 secoisolariciresinol and matairesinol are also precursors to mammalian lignans (Heinonen, et
38 al., 2001; Njastad, et al., 2014). Other plant lignans may be converted to secoisolariciresinol
39 and matairesinol through intermediate reactions and thereby contribute to enterodiol and
40 enterolactone synthesis (Heinonen, et al., 2001; Njastad, et al., 2014). There is a scarce
41 information about coumestrol metabolism but its transformation in the rumen is rather limited
42 (Njastad, et al., 2014). Further to their metabolism in the rumen, and during intestinal
43 absorption, phytoestrogens are conjugated mainly with glucuronic and sulfonic acids and
44 transferred to blood and milk as conjugates (Njastad, et al., 2014).

45 Phytoestrogens are found both in forages and concentrates of cows' diets. The
46 phytoestrogens content in forage depends mainly on the botanical composition, but also plant
47 part, maturity stage, season (Booth, et al., 2006; Hojer, et al., 2012; Kallela, Saastamoinen, &
48 Huokuna, 1987; Tsao, Papadopoulos, Yang, Young, & McRae, 2006). Therefore, the milk
49 phytoestrogen concentrations may be influenced by grassland management and dairy
50 management system (Adler, et al., 2014; Adler, Purup, Hansen-Moller, Thuen, & Steinshamn,

51 2015; Hojer, et al., 2012). The concentration of isoflavones was higher in cow diets that
52 contained red clover than other species (Andersen, et al., 2009; Hojer, et al., 2012; Njastad, et
53 al., 2014). This may affect organic milk isoflavone concentrations because, in the absence of
54 nitrogen fertilization of organic swards, UK organic dairy systems extensively rely on clover
55 inclusion in pastures and silages to achieve high sward productivity (AHDB, 2012; Soil
56 Association, 2018; Stergiadis, et al., 2015; Stergiadis, et al., 2012). The effect of dairy
57 management system on milk phytoestrogens concentrations has been observed in Finland,
58 Norway and Denmark (Adler, et al., 2015; Hoikkala, et al., 2007; Purup, 2005). Finnish organic
59 retail milk contained more equol than conventional milk (Hoikkala, et al., 2007). In Norway,
60 organic milk collected from farms' bulk-tanks had higher concentrations of formononetin,
61 daidzein, equol, genistein, secoisolariciresinol, enterodiol and enterolactone, than conventional
62 milk; moreover, milk produced during the indoor period contained more equol than milk from
63 the outdoor period, whereas the opposite was observed for enterolactone (Adler, et al., 2015).
64 In Denmark, the concentration of isoflavonoids in milk from organic dairy farms was higher
65 than milk from conventional farms; presumably because of the higher contribution of legumes
66 in organic dairy diets; notably, the content of equol in organic milk was approximately five
67 times higher (Purup, 2005).

68 Although results from Finland, Norway and Denmark demonstrate strong management
69 system and seasonal effects on isoflavone and lignan concentrations in milk, a similar
70 assessment in the UK has not been performed. Previous work on milk fatty acid profiles
71 demonstrates that composition of organic and conventional milk, may differ significantly
72 between countries, thus highlighting the necessity for milk composition assessments in
73 different countries separately (Butler, et al., 2011). The aim of the present work was (i) to
74 investigate, for the first time, the effect of, and interactions between, dairy management system
75 (including conventional, organic and free-range) and month, on the concentrations of lignans,

76 isoflavones and coumestans in milk purchased from retail outlets in the United Kingdom, and
77 (ii) assess the potential impact of consuming milk from different dairy management systems
78 on phytoestrogen intake of consumers.

79 **2. Materials and methods**

80 *2.1 Experiment/survey design*

81 Milk samples (n=120) representing four brands of conventional (CNV; n=48) milk and
82 four brands of organic (ORG; n=48) milk were collected monthly, over 12 months (March
83 2016 and February 2017), within an 8-km radius from the Whiteknights campus of the
84 University of Reading. Brands were selected to represent suppliers that offer both CNV and
85 ORG milk and have as high as possible market share. Two brands of milk certified as free-
86 range (FRG; n=24), which were the only two brands available when the survey took place,
87 were also monthly collected during the same sampling dates from dairies in Lancashire and
88 Gloucestershire. ORG milk was certified by Soil Association and FRG milk by the Free Range
89 Dairy Pasture Promise. Only whole, pasteurized and homogenized milk was collected; CON
90 and ORG milk was purchased from supermarkets while retail free-range milk was posted, in
91 cold conditions, from the Free Range Network to the University of Reading. Milk from CNV
92 and FRG systems were fat-standardised at approximately 35 and 37 g/kg milk, respectively;
93 this is a standard practice in the conventional UK dairy plants while standardization is not
94 performed in the organic UK dairy supply chain. At the day of collection, the commercial bottle
95 (typically made of high-density polyethylene) with the furthest “best before” date (hence the
96 most recent on the shelf) was purchased to maximize freshness of the collected sample. This
97 has been immediately transferred in cold conditions at the laboratories of the University of
98 Reading and was aliquoted in several 30-ml sterile, screw-top, leak-free polypropylene
99 containers and frozen at -20°C until analysis.

100 2.2 Milk analysis

101 One aliquote of 30-ml of each sample, packed with several ice packs into polystyrene
102 boxes and by using next day delivery in order to ensure samples remained frozen throughout
103 transport, was sent to Aarhus University. Upon delivery, samples were immediately stored at -
104 20°C and were analysed in the laboratory between January and May 2018. Quantitative
105 measurements were performed using the following lignans and isoflavones standards:
106 enterolactone, enterodiol, matairesinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol,
107 isolariciresinol, syringaresinol, medioresinol, pinoresinol, equol, naringenin, formononetin,
108 chrysin, genistein, daidzein, glycitein, coumestrol, purchased from Plantech (Berksher, UK).
109 The following isotope-labeled and deuterium-labeled internal standards were used: ¹³C₃-
110 enterolactone, ¹³C₃-enterodiol, equol D4 from Toronto Research Chemicals (Toronto, Canada)
111 and genistein-d4 and daidzein-d3 from Cambridge Isotop Laboratories, Inc. (Andover, MA,
112 USA). For the enzymatic hydrolysis, β-glucuronidase type H-1 from *Helix pomatia* was
113 purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used were of HPLC grade.

114 2.2.1 Preparation and storage of standards

115 All lignan standards were dissolved in pure acetonitrile and all isoflavones standards
116 were dissolved in pure methanol in concentration of 1 mg/mL and kept at -80 °C, except for
117 genistein-D4 and daidzein-d3 internal standards, which were dissolved in acetonitrile in
118 concentration 100 µg/mL and 60 µg/mL respectively. The working solutions of lignan internal
119 standards were prepared in concentration of 10 µg/mL of ¹³C₃-enterolactone, 5 µg/mL of ¹³C₃-
120 enterodiol and 50 µg/mL of equol-d4. For non-labeled standards, one working solution
121 contained all lignan and equol standards and another working solution contained other
122 isoflavones in the concentration of 400 ng/mL. These working solutions were used for
123 preparation of standard curves and spiking of milk samples. The working solution and the
124 standard curves were kept at -80 °C at all times.

125 *2.2.2 Milk sample preparation*

126 The milk samples (kept at -20 °C before the analyses) were incubated in water bath at
127 30 °C for 60 min and afterwards immediately shaken for 10 min. Five ml of milk sample was
128 transferred to 15 mL tube and 10 µL of the internal standards, ¹³C₃-enterolactone, ¹³C₃-
129 enterodiol, genistein-d4, daidzein-d3 and equol-d4 were added and mixed for 5 min. The
130 samples were then centrifuged for 20 min at 4 °C at 3,500 × g. One ml of the supernatant was
131 transferred to a new tube to which 0.5 mL of enzymes were added. Further sample hydrolyses
132 and clean-up were performed according to Norkov, Olsen, Tjonneland, Bolvig, Laerke, &
133 Knudsen (2015) with minor modification. Lignans and isoflavones were eluted with 200 µL of
134 acetonitrile from C18 plates and diluted with 600 µL of MilliQ water and analyzed using LC-
135 MS/MS.

136 *2.2.3 LC-MS/MS equipment and method*

137 The LC-MS/MS measurements were performed on microLC 200 series from
138 Eksigent/AB Sciex (Redwood City, CA, USA) and QTrap 5500 mass spectrometer from AB
139 Sciex (Framingham, MA, USA) according to Norkov et al. (2015). The compound dependent
140 parameters were optimized for each compound by syringe infusion of pure standard and shown
141 in Appendix (Table A1). The data analysis was performed in Analyst software 1.6.2 from AB
142 Sciex (Framingham, MA, USA).

143 *2.2.4 Calibration curves and quantification*

144 Calibration curves had 7-12 points depending on the analyte. The mixture of pure
145 standards were prepared in 25% acetonitrile in the range of 0.0244 – 100 ng/mL for lignans,
146 0.39 – 200 ng/mL for equol (see Appendix, Figure A1), and 0.00977 – 5 ng/mL for all other
147 isoflavones (see Appendix, Figure A2). The final concentrations were 25 ng/mL for ¹³C₃-
148 enterolactone was, 12.5 ng/mL for ¹³C₃-enterodiol, 60 ng/mL for daidzein D3, 30 ng/mL for

149 genistein-d4 and 200 ng/mL for equol-d4. The analyte/internal standard concentration ratio
150 was plotted against the analyte/internal standard peak area ratio as a linear regression curve
151 with 1/x weighting. The quantification of the lignans, enterolactone, matairesinol and
152 pinoresinol was performed using ¹³C₃-enterolactone as internal standard; and that of enterodiol,
153 hydroxymatairesinol, secoisolariciresinol, lariciresinol, isolariciresinol, syringaresinol and
154 medioresinol using ¹³C₃-enterodiol as internal standard. The quantification of isoflavones,
155 daidzein and glycitein was performed using daidzein-d3 as internal standard; and that of
156 genistein, naringenin, formononetin, chrysin and coumestrol using genistein-d4 as internal
157 standard. Equol was quantified using equol-d4 as internal standard. The lower limit of
158 quantitation (LLOQ) was accepted as the lowest standard on the calibration curve if the analyte
159 response was at least 5 times the response of the blank sample. The highest standard defined
160 the upper limit of quantitation (ULOQ). All calibration curves showed good linearity
161 throughout the used range of concentration with LLOQ accuracy varying from 88 to 110 %
162 and precision below 20%, and ULOQ accuracy from 90 to 105% and precision below 15%.
163 The LLOQ and ULOQ and the corresponding regression coefficients for each isoflavone is
164 listed in the Appendix (Table A2), and for lignans in (Norskov, et al., 2015). The representative
165 chromatogram of the milk sample, as well the extracted ion chromatograms of each lignan and
166 isoflavone, are shown in the Appendix (Figure A3 and A4, respectively).

167 *2.2.5 Method validation*

168 Method was validated by spiking internal standards and lignan and isoflavone standards
169 in the beginning of the sample preparation procedure (addition of standards to 5 mL of milk
170 sample) to the experimental milk samples containing the lowest possible concentration of
171 lignans and isoflavones; recovery was then calculated using the internal standard procedure, as
172 described above. The recovery of enterolactone and enterodiol was 105 % ± 3% and that of
173 plant lignans (matairesinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol,

174 isolariciresinol, syringaresinol, medioresinol and pinoresinol) was $75 \% \pm 6\%$. The recovery
175 of equol was $112 \% \pm 3\%$ and the recovery of other isoflavones were $95 \% \pm 10\%$. Precision
176 and intra-batch variation (based on 5 replicated measurements) were within 15%. Further, the
177 validation included the detection of possible lignans and isoflavones of interest in the enzyme
178 mixture used for hydrolyses. Only trace amounts of formononetin (0.004 ng/mL), glycitein
179 (0.006 ng/mL), naringenin (0.04 ng/mL), genistein (0.05 ng/mL), daidzein (0.009 ng/mL),
180 enterolactone (0.001 ng/mL), secoisolariciresinol (0.002 ng/mL) and lariciresinol (0.03 ng/mL)
181 were detected, and no coumestrol, equol, chrysin, isolariciresinol, enterodiol, matairesinol,
182 hydroxymatairesinol, syringaresinol, medioresinol and pinoresinol were detected.

183 *2.3 Statistical analysis*

184 Linear mixed effects models in GenStat 17th Edition (VSN International, UK) were
185 used for the Analysis of Variance (ANOVA) (Residual maximum likelihood analysis; REML;
186 (Gilmour, Thompson, & Cullis, 1995)). Fixed factors were the management system
187 (Conventional, CNV; Organic, ORG; Free-Range, FRG) and month (March 2016 - February
188 2017). To investigate the effect of the interaction between management system and month, a
189 sub-set including only ORG and CNV milk has been created (excluding FRG milk) and a
190 REML analysis was carried out using management system, month and their interaction as fixed
191 factors. Every milk sample was given a unique milk ID (representing the combination of
192 brand/retailer and management) and this was used as random factor in both REML analyses.
193 The analysis derived and P-value and the effect of the main treatments was declared significant
194 at $P < 0.05$; tendencies were declared at $0.05 < P < 0.10$. Normality plots were used for
195 performing the residual diagnostics of the model and data did not deviate from normality.
196 Follow up pairwise comparison of means ($P < 0.05$) in cases that the effect of a fixed factors
197 showing a significant effect were performed using Fisher's Least Significant Difference test.

198

199

3. Results

200 *3.1 Overall milk concentrations of phytoestrogens*

201 The concentration of plant isoflavones in milk was 5-8 times higher compared with
202 plant lignans and coumestrol, averaging 5.95 ng/mL (ranging 1.21-12.85 ng/mL) across the
203 management systems; whereas the average plant lignan concentration was 0.90 ng/mL (ranging
204 0.51-1.64 ng/mL). The average equol and enterolactone concentrations was 203.1 ng/mL
205 (ranging 4.3-794.4 ng/mL), and 61.9 ng/mL (ranging 32.9-138.9 ng/mL), respectively, across
206 the management systems.

207 *3.2 Dairy management system and milk phytoestrogens composition*

208 The effect of dairy management system was significant for secoisolariciresinol,
209 matairesinol, lariciresinol, total plant lignans, daidzein, genistein, formononetin, naringenin,
210 total plant isoflavones, equol, total isoflavones and coumestrol (Table 1). ORG milk contained
211 more secoisolariciresinol (+0.06 and +0.04 ng/ml milk), matairesinol (+0.05 and +0.03 ng/ml
212 milk), lariciresinol (+0.14 and +0.14 ng/ml milk), total plant lignans (+0.26 and +0.16 ng/ml
213 milk), daidzein (+1.74 and +1.72 ng/ml milk), genistein (+1.49 and +1.47 ng/ml milk),
214 formononetin (+1.01 and +1.01 ng/ml milk), naringenin (+0.13 and +0.12 ng/ml milk), total
215 plant isoflavones (+4.64 and +4.69 ng/ml milk), equol (+347 and +345 ng/ml milk), total
216 isoflavones (+352 and +349 ng/ml milk) and coumestrol (+0.35 and +0.37 ng/ml milk), than
217 FRG and CNV milk (Table 1). The differences in individual lignan or isoflavonoid
218 concentrations between CNV and FRG milk was not significant (Table 1).

219 *3.3 Sampling month and milk phytoestrogens composition*

220 The effect of month was significant for secoisolariciresinol, matairesinol, lariciresinol,
221 hydroxymatairesinol, total plant lignans, enterolactone, enterodiol, total mammalian lignans,
222 daidzein, genistein, formononetin, total plant isoflavones and total isoflavones (Table 2). Milk
223 concentrations of secoisolariciresinol, matairesinol, lariciresinol and total plant lignans were

224 increased during May-October than during March-April and November-February, with
225 differences between individual months not always being statistically significant (Table 2). In
226 contrast, concentrations of hydroxymatairesinol in milk were higher in March and December-
227 February, when compared with the period April-November, but differences between individual
228 months were not always statistically significant (Table 2). Milk concentrations of
229 enterolactone, total mammalian lignans and total lignans were increased during May-August,
230 than during March and December-February and had intermediate values during the other
231 months (Table 2). Milk concentrations of enterodiol in milk were increased during May and
232 July-October, than during April, November and February and had intermediate values during
233 the other months (Table 2). Milk concentrations of daidzein in milk were increased during
234 August and October, although this was only statistically significant when compared with June
235 and Jan-Feb (Table 2). Milk concentrations of genistein in milk were increased during March-
236 April and October-November, when compared with the period May-June; their values were
237 intermediate during the other months (Table 2). Milk concentrations of glycitein were higher
238 during March-May, than during the period of June-July and November and had intermediate
239 values during the other months (Table 2). Concentrations of milk total plant isoflavones had
240 highest values during March, lowest values in June and intermediate values during the other
241 months, but differences between individual months was not always statistically significant.
242 Milk concentrations of formononetin were increased during March, August and November,
243 when compared with May-June; while its values were intermediate during the other months
244 (Table 2). Milk equol and total isoflavone concentrations were increased during March and
245 October-February than during April-September; but differences between individual months
246 were not always statistically significant (Table 2).

247 *3.4 Interactions between management system and sampling month on milk phytoestrogens*
248 *composition*

249 The effects of management system \times month interaction was significant for daidzein,
250 genistein, formononetin, equol and total isoflavones (Figure 1), secoisolariciresinol,
251 matairesinol, lariciresinol, total plant lignans, enterolactone, enterodiol, total mammalian
252 lignans and total lignans (Figure 2). Milk daidzein, genistein, formononetin, equol and total
253 isoflavone concentrations were higher in ORG than in CNV milk, throughout the year, but the
254 extent of the differences was fluctuating throughout the season; the highest differences were
255 observed in November-February, for equol and total isoflavones; in August-October, for
256 daidzein; August-November for Genistein; and November and March for formononetin
257 (Figure 1). The management system \times month interaction did not have a significant effect on
258 milk concentrations of hydroxymatairesinol, glycitein, naringenin and coumestrol. Milk
259 concentrations of secoisolariciresinol and lariciresinol were higher in ORG than in CNV milk
260 in June-October (Figure 2). Milk concentrations of matairesinol increased in ORG milk during
261 May-September, when compared with CNV milk, while the same was observed for total plant
262 lignans, by also extending this significant difference to April (Figure 2). Concentrations of
263 enterolactone, mammalian lignans and total lignans were increased in ORG milk, when
264 compared with CNV milk, during June, August and December-February (Figure 2). Milk
265 concentrations of enterodiol were higher in ORG than CNV milk during March, May-August
266 and January (Figure 2).

267 **4. Discussion**

268 *4.1 Overall milk concentrations of phytoestrogens*

269 In line with previous studies, equol and enterolactone were the main phytoestrogens in
270 bovine milk (Adler, et al., 2014; Adler, et al., 2015; Andersen, et al., 2009; Hojer, et al., 2012;
271 Njastad, et al., 2014; Steinshamn, et al., 2008), possibly because these are the end products of
272 mammalian metabolism of plant isoflavones and lignans, and in particular formononetin,
273 daidzein, secoisolariciresinol and matairesinol (Njastad, et al., 2014). Enterodiol (another

274 mammalian lignan) was found in lower concentrations, as this is an intermediate in the process
275 of enterolactone synthesis from plant lignans (Njastad, et al., 2014). The concentration of plant
276 lignans secoisolariciresinol and matairesinol was lower compared with other studies (Adler, et
277 al., 2014; Adler, et al., 2015; Steinshamn, et al., 2008), whereas the concentration of plant
278 isoflavones, genistein, daidzein and formononetin was comparable (Adler, et al., 2015;
279 Steinshamn, et al., 2008). Milk coumestrol concentration was low, but similar to previous work
280 (Adler, et al., 2014; Adler, et al., 2015). The high variation in the concentration of equol and
281 enterolactone between different studies imply that milk phytoestrogen concentrations may be
282 influenced by a combination of genetics, diets and/or other management practices. Differences
283 between studies are also because milk samples are collected at contrasting stages of the dairy
284 supply chain, e.g. retail milk in the current work and milk from individual cows or farm bulk-
285 tank in other studies (Adler, et al., 2014; Adler, et al., 2015; Steinshamn, et al., 2008). Milk
286 chemical composition, including phytoestrogens, is strongly influenced by animal genetics and
287 farm diets, when milk is collected from individual cows or farms. In contrast, milk collected at
288 retail level represents the average composition of milk from each management system,
289 resulting from mixing milk from a large numbers of farms prior to processing and packaging.
290 This dilutes any extreme values from individual cows or herds that may be fed diets with strong
291 effect on milk phytoestrogens concentrations. To our knowledge, this is the first study to
292 measure lariciresinol, hydroxymatairesinol, glycitein and naringenin in bovine retail milk.

293 *4.2 Dairy management system and milk phytoestrogens composition*

294 *4.2.1 Organic milk*

295 Differences in the concentrations of individual lignans and isoflavones between ORG
296 and CNV milk might be caused by the contrasting diets in these management systems. The
297 higher concentrations of secoisolariciresinol in ORG milk are in agreement with Adler et al.
298 (2015), who compared organic and conventional milk under short- or long- term access to

299 pasture. White and red clover are commonly used in ORG dairy diets in the UK (AHDB, 2012;
300 Soil Association, 2018; Stergiadis, et al., 2015; Stergiadis, et al., 2012). Fresh or ensiled dietary
301 clover increases feed transfer rates from the rumen (Dewhurst, Evans, Scollan, Moorby, Merry,
302 & Wilkins, 2003; Stergiadis, et al., 2018), thus subsequently reducing the time feed is available
303 to rumen microbes and the conversion of secoisolariciresinol (and other plant lignans) to
304 enterolactone and enterodiol.

305 The higher concentration of secoisolariciresinol in ORG compared to CNV and FRG
306 milk is in agreement with Adler et al. (2015). However, management system did not affect
307 concentrations of enterodiol and enterolactone (mammalian lignans), as previously reported
308 (Njastad, et al., 2014), nor the sum of lignans, in the present work. This is in contrast with
309 Adler et al. (2015), who found higher concentrations of enterolactone and enterodiol and
310 lignans in ORG milk. The higher presence of secoisolariciresinol and matairesinol, precursors
311 to enterolactone, in ORG milk in this study, may indicate a higher secoisolariciresinol dietary
312 supply from the clover-based ORG dairy diets (AHDB, 2012; Soil Association, 2018). In
313 addition, grain lignans are bound to cell wall macromolecules of the bran (Fardet, 2010) and
314 have low bioavailability, thus potentially reducing their conversion to enterolactone. Therefore,
315 the bioavailability of secoisolariciresinol may be lower in high-grain CNV diets, typical in the
316 UK CNV dairy systems (Stergiadis, et al., 2012), and this reduces their concentrations in milk.
317 In contrast to the present work, Njastad et al. (2014) found similar concentrations of
318 secoisolariciresinol, and matairesinol in ORG and CNV milk. The contrasting effects between
319 studies may result from the variant composition of concentrates in CNV diets, e.g. made up of
320 different grains and thereby altering supply of plant lignans, which are precursors to
321 enterolactone. For example, plant lignans in wheat, which is the main grain used in UK dairy
322 diets, is higher than in oat and barley (Smeds, et al., 2007), which are also used but in lesser

323 extent. However, background information of grain contribution and composition in the dairy
324 diets of different management systems was not available in the current work.

325 The concentration of hydroxymatairesinol and glycitein was similar between the
326 management systems, potentially because hydroxymatairesinol is predominant lignan in oats
327 and barley (Smeds, et al., 2007); feeds that contribute less in cow diets than other grains (e.g.
328 wheat, maize) and only minor differences are expected between diets in the different
329 management systems. Given that hydroxymatairesinol in milk may originate from dietary
330 hydroxymatairesinol, that has been transferred to milk, or synthesized from matairesinol
331 through phase I metabolism (Niemeyer, Honig, Kulling, & Metzler, 2003).

332 Similar to the present work, Adler et al. (2015) showed higher concentration of
333 isoflavones, daidzein, genistein, and formononetin in ORG, than CNV, milk. Legumes,
334 including white and red clover which are extensively used in ORG dairy diets (AHDB, 2012;
335 Soil Association, 2018; Stergiadis, et al., 2015; Stergiadis, et al., 2012), are richer in
336 isoflavones (including daidzein, genistein and formononetin) and coumestrol, than grass and
337 grains (Adler, et al., 2014; Kuhnle, et al., 2008). This increases their dietary intakes and the
338 subsequent amounts that are absorbed and transferred into milk (Njastad, et al., 2014). Previous
339 studies in Finland, France and Norway demonstrated that the high concentration of isoflavones,
340 and especially equol, in ORG milk were because of higher intakes of ensiled or fresh red clover
341 (Adler, et al., 2014; Adler, et al., 2015; Andersen, et al., 2009; Antignac, Cariou, Le Bizec, &
342 Andre, 2004; Hoikkala, et al., 2007; Hojer, et al., 2012; Steinshamn, et al., 2008). Red clover
343 is rich in daidzein, formononetin, and biochanin A, which are precursors of equol (Mustonen,
344 et al., 2009), and the most common legume for silage making in the ORG dairy farms in the
345 UK (AHDB, 2012). Equol concentrations in ORG and CNV milk in the study of Hoikkala et
346 al. (2007) were very similar (411 and 62 ng/ml, respectively) to those measured in the current

347 work (411 and 64 ng/ml, respectively), although those in retail milk in the study of Antignac
348 et al. (2004) were lower (191 and 36 ng/mL, respectively).

349 ORG dairy cow diets also have a higher forage:concentrate ratio than CNV diets
350 throughout the year (Soil Association, 2018; Stergiadis, et al., 2012). Steinshamn et al. (2008)
351 found that concentrate supplementation reduces the intake of most phytoestrogens, including
352 equol, biochanin A and daidzein. This may be an additional reason for their lower concentration
353 in CNV milk, as the lower intakes would reduce their outputs in milk, as well as the amounts
354 of *in vivo* synthesised equol (Njastad, et al., 2014).

355 *4.2.2 Free-range milk*

356 In the present work, the phytoestrogen concentrations did not differ between CNV and
357 FRG milk, which is probably due to small feeding differences between these two management
358 systems. Similar findings, and extensive potential explanations, have been recently published
359 for milk fatty acids, which are also strongly influenced by cow diet (Stergiadis, Berlitz, Hunt,
360 Garg, Givens, & Kliem, 2019). Farm management practices have not been recorded in the
361 present work, and there is limited information available to describe FRG management, but
362 similar diets, in terms of forage:concentrate ratio and forage species used, between CNV and
363 FRG farms may explain the similar milk concentrations of lignans and isoflavonoids.

364 *4.3 Seasonal effect on milk phytoestrogens composition*

365 In UK farms that use grazing as a feeding practice, cows would typically turnout to
366 pasture around late March-beginning of April and access will be provided until approximately
367 late-October, with grazing intake being maximum between May and August (AHDB, 2011).
368 In late October-early November, cows would be taken indoors, and fed with conserved forages
369 and concentrates (AHDB, 2011).

370 In the present work, milk lignan concentrations (including enterolactone,
371 secoisolariciresinol, and matairesinol and lariciresinol), were higher during the

372 outdoors/grazing period, as previously shown in Norway for enterolactone, secoisolariciresinol
373 and matairesinol (Adler, et al., 2015). This may result from the higher concentrations and
374 bioavailability of lignans in the pastures, which contribute more in cow diets during the grazing
375 season. In the present work, 40% of samples were ORG and cows were expected to graze
376 pastures rich in legumes, and in particular clover (AHDB, 2012; Soil Association, 2018;
377 Stergiadis, et al., 2015; Stergiadis, et al., 2012). In contrast, the higher concentration of
378 hydroxymatairesinol was higher during indoor period, which is expected as the main source of
379 hydroxymatairesinol in cow diets are the concentrates rather than the forages.

380 Milk concentrations of sum of and individual isoflavones (including daidzein,
381 genistein, formononetin, naringenin and equol) were higher during the typical indoor season,
382 thus aligning with previous results for equol, daidzein, formononetin and the sum of
383 isoflavones, although previous findings were not always statistically significant (Adler, et al.,
384 2015). It is possible that the contribution of clover, which is rich in isoflavones (Steinshamn,
385 et al., 2008), is higher in silages than in pasture because silage swards are harvested when
386 clover biomass contribution is relatively high. Milk isoflavone concentration is strongly
387 influenced by silage botanical composition (Hojer, et al., 2012), something that might have
388 contributed to seasonal differences in the present work. Red clover is richer in formononetin,
389 a precursor to equol, than other legumes such as white clover, timothy, meadow fescue and
390 birdsfoot trefoil (Hojer, et al., 2012). Given that red clover is a typical legume for silage making
391 in the UK, while pastures contain substantial amounts of white clover (AHDB, 2012; Soil
392 Association, 2018), the grass/red-clover silage (fed during the indoor period) would provide
393 more dietary daidzein, genistein, formononetin and biochanin A than grass/white-clover
394 pastures (Steinshamn, et al., 2008). In addition, the pasture and silage concentrations of
395 daidzein, genistein, formononetin and biochanin A are influenced by plant growth stage, and
396 the biomass leaf:stem ratio (Booth, et al., 2006; Hojer, et al., 2012; Tsao, et al., 2006).

397 Isoflavones are in higher concentrations in leaves than in stems (Tsao, et al., 2006) and, given
398 that clover is harvested for silage making at strategic times when leaf:stem ratio is higher than
399 the typical leaf:stem ratio when cows are grazing, clover-based silages would provide more
400 dietary isoflavones than grass-clover pastures. The higher contribution of soybean meal, which
401 is rich in daidzein and genistein, in cow diets during the indoor period in the CNV and FRG
402 systems may explain the increase in milk isoflavone concentrations. Genistein, glycitein,
403 daidzein showed similar, but not as strong, seasonal patterns as equol. This indicates that equol
404 concentrations represent the combined effect of clover silage and soybean supplementation,
405 while one of these factors may have less impact in the other isoflavones.

406 *4.4 Interaction between management system and season on milk phytoestrogens composition*

407 Seasonal variation of phytoestrogens was stronger in the ORG, than in CNV, milk. This
408 is because the main driver for milk phytoestrogen concentrations is cow diet (Adler, et al.,
409 2014; Njastad, et al., 2014), and dairy diets are more diverse throughout the year in ORG than
410 in CNV systems. In ORG herds, cows ought to graze at least 60% of their dry matter intake
411 during the grazing season, and the typical grass/clover swards have variant contribution of
412 clover between different months (AHDB, 2012; Soil Association, 2018). Cows in CNV herds
413 also typically graze between April-October, but the intakes of grazed forage would be lower
414 than in ORG cows (Stergiadis, et al., 2012), while clover is not commonly used, and the relative
415 impact on seasonal variation will be less pronounced. In addition, highly-intensive dairy farms
416 (Stergiadis, et al., 2012), which also contribute to the CNV retail milk pool, do not allow cows
417 to graze and offer similar diets throughout the year (based on conserved forage and
418 concentrates), thus further reducing the seasonal effect (Stergiadis, et al., 2012).

419 The concentrations of secoisolariciresinol, matairesinol and lariciresinol were higher in
420 ORG, than in CNV milk, during parts of the grazing season, but not during the indoor season.
421 The main driver of the concentrations of these lignans in milk is their dietary intake (Adler, et

422 al., 2015). Clover-containing pasture can be a main source (Adler, et al., 2014), and the higher
423 pasture intakes of ORG cows during the grazing season may explain this finding. This
424 difference is reduced in winter, when both ORG and CNV cows are housed indoors and receive
425 conserved forages and concentrates; although ORG silage may still supply more clover than
426 CNV silage.

427 Concentrations of daidzein, genistein, formononetin, equol and sum of isoflavones
428 were higher in the ORG, than in CNV, throughout the year, and in particular during the indoor
429 season. Provided that red clover is the main driver for milk equol concentrations, and ORG
430 silages are made mainly using red clover, while ORG grazing swards may also contain
431 substantial amounts of white clover (AHDB, 2012), it is expected that winter diets may contain
432 more red clover. This increases the supply of daidzein, formononetin and biochanin A
433 (Steinshamn, et al., 2008) for equol synthesis in the rumen during the indoor period. In addition,
434 a higher contribution of overall clover and leaf:stem ratio, both factors known to increase
435 isoflavones supply (Adler, et al., 2014; Tsao, et al., 2006), is expected in the ensiled forage
436 than in grazed sward, because harvest for silage takes place when there is a higher clover
437 biomass and leaf:stem ratio, compared to that grazed by cows in grass-clover pastures.

438 Enterolactone concentrations were not influenced by the management system but ORG
439 milk contained more enterolactone during summer, similarly to other plant lignans, but less
440 enterolactone during winter, when compared with CNV milk. The intakes of plant lignans,
441 which are precursors to enterolactone, potentially decrease at higher rates during winter period
442 in ORG diets; while rumen microbes may become more efficient in metabolising feed
443 phytoestrogens over time (Njastad, et al., 2014).

444 *4.5 Potential impact of consuming milk from different dairy management systems on*
445 *phytoestrogen intakes of UK consumers*

446 In the most recent National Diet and Nutrition Survey in the UK (Bates, et al., 2014),
447 liquid milk consumption (average; including whole, semi-skimmed and skimmed milk) for
448 males and females was 275 g/day for children 1.5-3.0 years, 187 g/day for children 4-10 years,
449 110 g/day for teenagers 11-18 years, 125 g/day for adults 19-64 years, and 181 g/day for adults
450 over 65 years. Antignac et al. (2004) showed that whole and skim milk have similar
451 phytoestrogen concentrations in France, and we assume that phytoestrogen profile measured
452 in whole milk in the present work will represent the profiles of other available retail liquid milk
453 in the UK (semi-skimmed, skimmed). Therefore, under the current intakes of liquid milk
454 (Bates, et al., 2014), a change from CNV to ORG milk will increase the intakes of equol by
455 95.3 µg/day in children 1.5-3.0 years (from 17.5 to 112.8 µg/day), 68.7 µg/day in children 4-
456 10 years (from 12.6 to 81.3 µg/day), 49.3 µg/day in teenagers 11-18 years (from 9.0 to 58.3
457 µg/day), 47.3 µg/day in adults 19-64 years (from 8.7 to 56.0 µg/day), and 64.0 µg/day in adults
458 over 65 years (from 11.7 to 75.7 µg/day). Across all ages and genders, a change from CNV to
459 ORG liquid milk would also increase the intakes of secoisolariciresinol by 0.01 µg/day (from
460 0.03 to 0.04 µg/day); matairesinol by 0.01 µg/day (from 0.02 to 0.03 µg/day); lariciresinol by
461 0.03 µg/day (from 0.06 to 0.09 µg/day); daidzein by 0.32 µg/day (from 0.18 to 0.50 µg/day);
462 genistein by 0.27 µg/day (from 0.16 to 0.43 µg/day); formononetin by 0.18 µg/day (from 0.02
463 to 0.20 µg/day); naringenin by 0.03 µg/day (from 0.03 to 0.06 µg/day) and coumestrol by 0.06
464 µg/day (from 0.02 to 0.08 µg/day).

465 The most substantial effect of switching to ORG milk on phytoestrogen intakes is
466 therefore observed for equol, while differences in intakes of other phytoestrogens are rather
467 low (<32 µg/day). Potential health benefits from equol and other phytoestrogen intake,
468 including lower risk for type-2 diabetes, cardiovascular disease, and hormone-dependent
469 cancers, action against osteoporosis and metabolic syndrome and reduction of menopausal
470 symptoms have been discussed in systematic reviews (Adlercreutz, 2007; Fardet, 2010;

471 Jungbauer & Medjakovic, 2014; Leitzmann, 2016). A meta-analysis of prospective cohort
472 studies, and a systematic review, established that for every 10 mg/day increase in the intake of
473 phytoestrogens, there is a 5% decrease on cardiovascular disease risk (Leitzmann, 2016; Wang,
474 Ouyang, Liu, & Zhao, 2014). However, the effects of phytoestrogens on human health have
475 not been sufficiently studied for the development of nutritional recommendations (Leitzmann,
476 2016). Although ORG milk contained more of the individual isoflavones and lignans,
477 nutritional recommendations and larger cohort and epidemiological studies to enlighten the
478 potential effects of phytoestrogens in human health (and the relative amounts required) are not
479 available. Therefore, in the present work it is not possible to conclude on any potential
480 implications of these differences on human health.

481 **5. Conclusion**

482 Organic retail milk had higher concentrations of the lignans secoisolariciresinol,
483 matairesinol, and lariciresinol, the isoflavones daidzein, genistein, formononetin, naringenin
484 and equol, and the coumestant coumestrol, when compared with milk from conventional or
485 free-range systems. There was a significant effect of management system on isoflavones and
486 coumestrol throughout the year but the effect on lignans was significant only during the typical
487 UK grazing season. In the present work, milk was collected at retail outlets and collecting
488 detailed information on dairy practices at farm level was not possible (beyond the label
489 certification). However, differences in phytoestrogen composition may be a consequence of
490 differing cow diets, and most likely an effect of the higher pasture and clover intakes in cow
491 diets in organic systems. The phytoestrogen composition did not differ between free-range and
492 conventional milk, but free-range milk had more secoisolariciresinol in August. Consuming
493 organic milk would increase intakes of lignans, isoflavones and coumestants but any influence
494 on human health as a result of these differences cannot be concluded from the results of the
495 present work.

496

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617

Table 1

Effect of dairy management system on the concentrations of lignans and isoflavones (means and SE) in retail milk samples collected monthly over 12 months.

Phytoestrogens (ng/ml)	Management System ^a				ANOVA P-values ^b
	CNV	ORG	FRG	SE	
Plant lignans					
Secoisolariciresinol	0.14 ^B	0.20 ^A	0.16 ^B	0.011	***
Matairesinol	0.12 ^B	0.17 ^A	0.14 ^{AB}	0.009	*
Lariciresinol	0.34 ^B	0.49 ^A	0.34 ^B	0.016	**
Hydroxymatairesinol	0.18	0.18	0.23	0.012	ns
Sum of plant lignans	0.78 ^B	1.03 ^A	0.87 ^B	0.030	**
Mammalian lignans					
Enterolactone	61.8	62.4	59.2	2.43	ns
Enterodiol	0.33	0.35	0.33	0.017	ns
Sum mammalian lignans	62.2	62.7	59.5	2.44	ns
Sum of lignans	63.0	63.7	60.4	2.45	ns
Plant isoflavones					
Daidzein	0.95 ^B	2.69 ^A	0.96 ^B	0.070	***
Genistein	0.83 ^B	2.32 ^A	0.85 ^B	0.078	***
Glycitein	2.07	2.34	1.97	0.118	ns
Formononetin	0.08 ^B	1.10 ^A	0.09 ^B	0.029	***
Naringenin	0.17 ^B	0.30 ^A	0.18 ^B	0.015	**
Sum of plant isoflavones	4.11 ^B	8.74 ^A	4.05 ^B	0.251	***
Mammalian isoflavones					
Equol	63.6 ^B	411.1 ^A	66.4 ^B	12.96	***
Sum of isoflavones	67.7^B	419.8^A	70.4^B	13.12	***
Plant coumestants					
Coumestrol	0.10 ^B	0.45 ^A	0.08 ^B	0.017	***

^a CNV = conventional (n=48), ORG = organic (n=48), FRG = free-range (n=24)

^b ***, P < 0.001; **, P < 0.01; *, P < 0.05; †, 0.05 < P < 0.10 (trend); ns, P > 0.10.

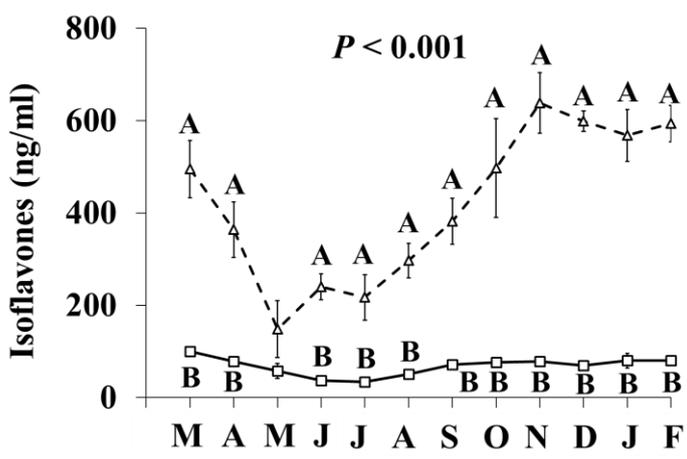
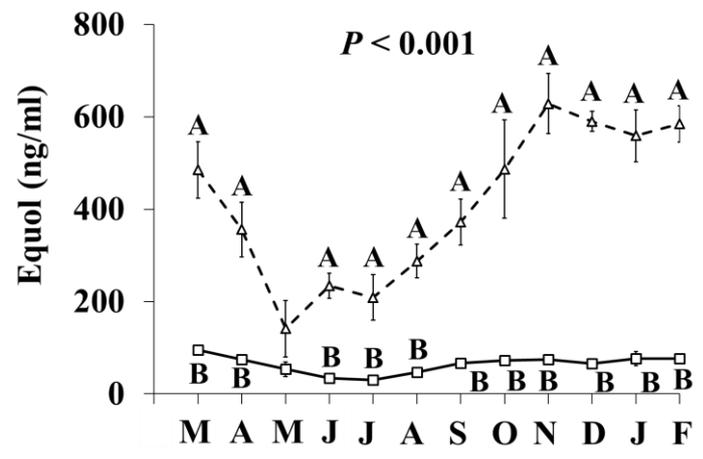
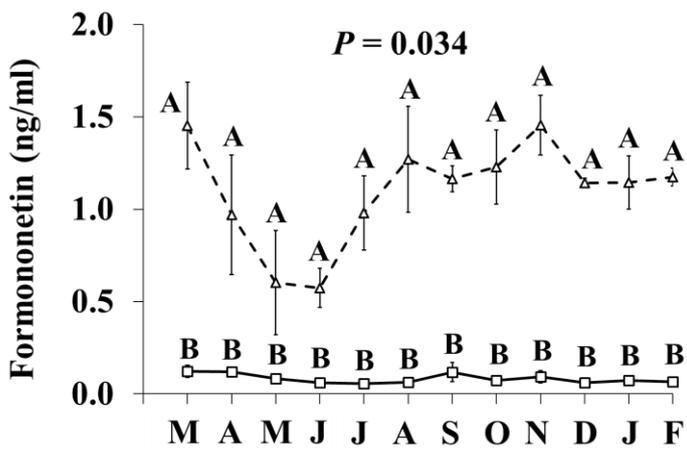
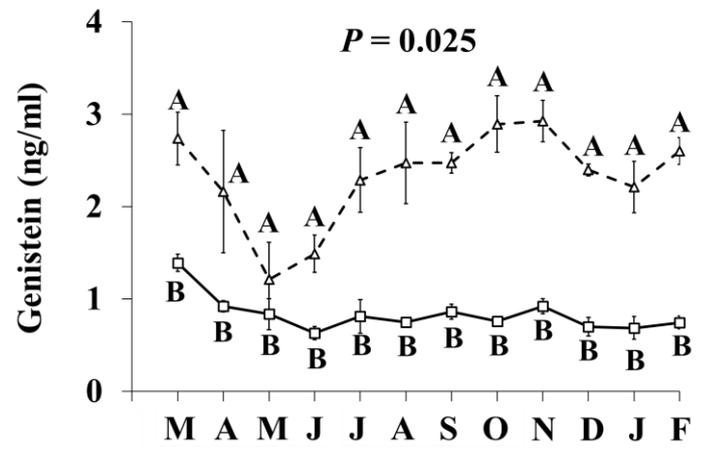
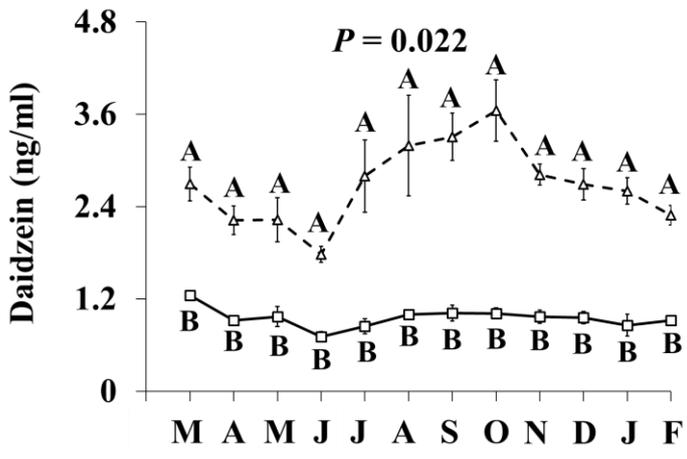
Different upper case letters within a row indicate significant differences between management system means (Fisher's Least Significant Difference test; P < 0.05)

Table 2

Effect of sampling month on the concentrations of lignans and isoflavones (means and SE) in retail milk samples collected monthly over 12 months

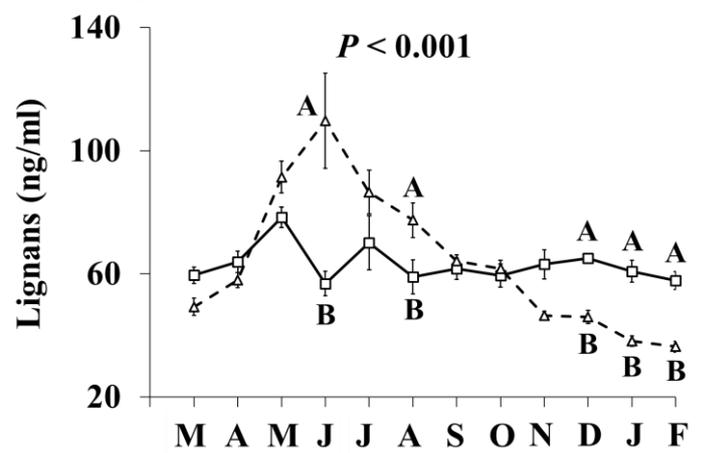
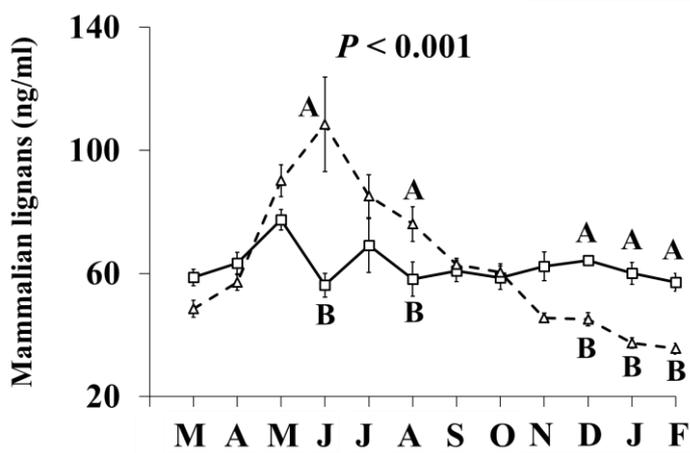
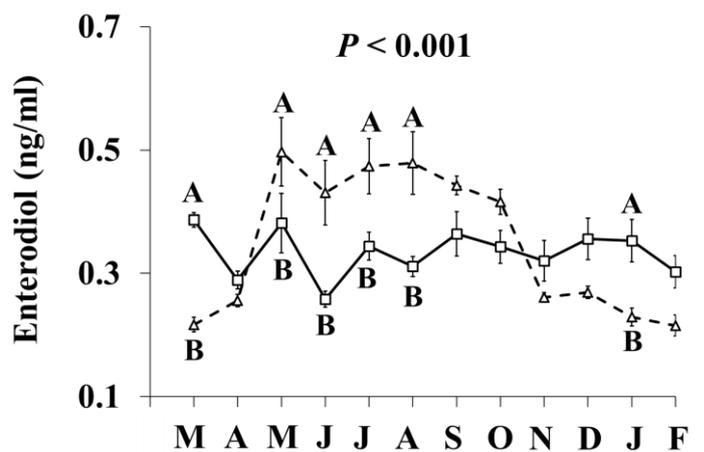
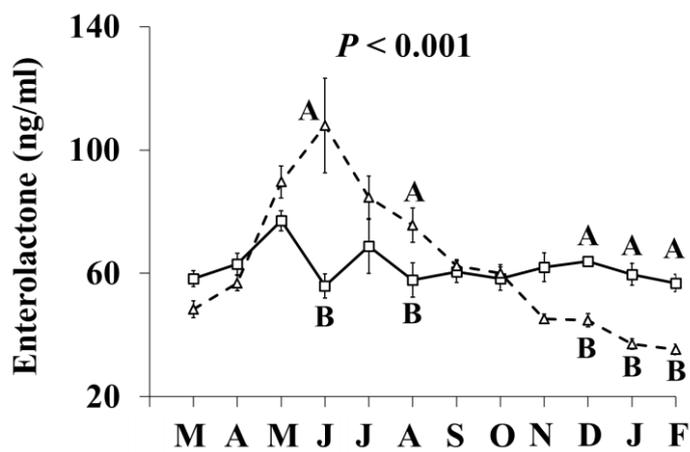
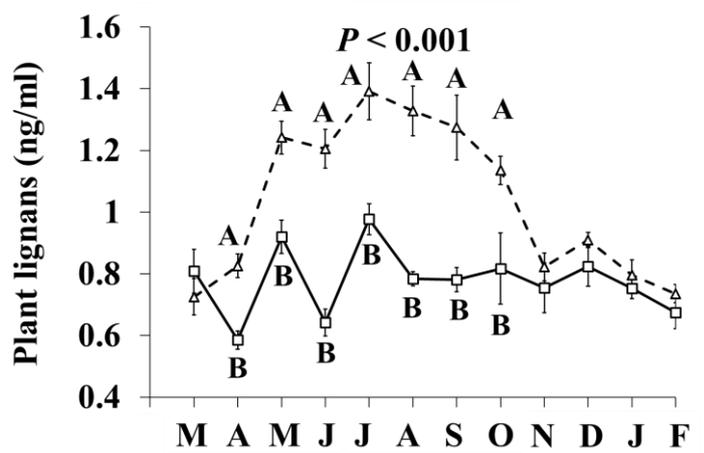
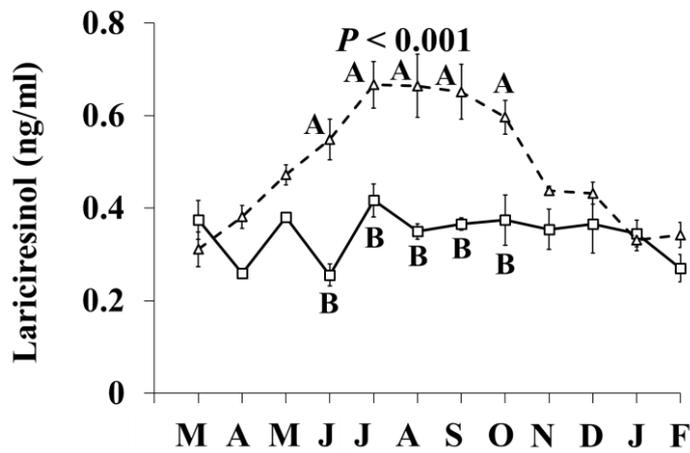
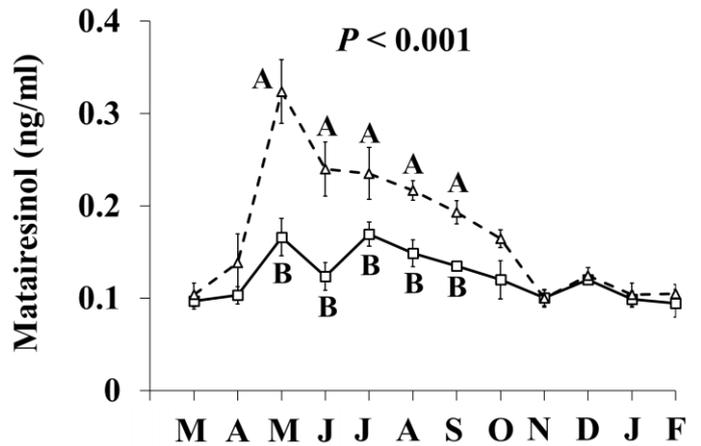
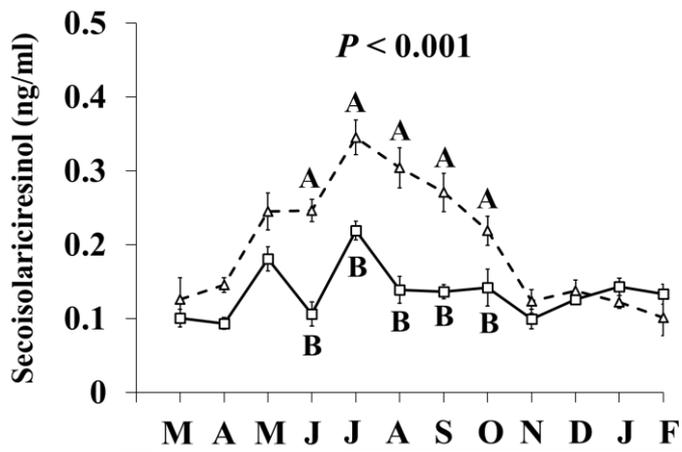
Phytoestrogens (ng/ml)	Month ^a												SE	ANOVA P-values ^b
	March	April	May	June	July	August	September	October	November	December	January	February		
Plant lignans														
Secoisolariciresinol	0.11 ^E	0.12 ^E	0.22 ^{AB}	0.17 ^C	0.26 ^A	0.24 ^A	0.20 ^{BC}	0.17 ^{CD}	0.11 ^E	0.13 ^E	0.13 ^{DE}	0.12 ^E	0.016	***
Matairesinol	0.10 ^D	0.12 ^{CD}	0.23 ^A	0.18 ^B	0.19 ^B	0.18 ^B	0.17 ^B	0.14 ^C	0.10 ^{CD}	0.12 ^{CD}	0.10 ^D	0.11 ^{CD}	0.013	***
Lariciresinol	0.34 ^{DEF}	0.32 ^{EF}	0.41 ^{BC}	0.39 ^{CD}	0.50 ^A	0.48 ^A	0.50 ^A	0.47 ^{AB}	0.39 ^{CD}	0.38 ^{CDE}	0.33 ^{EF}	0.30 ^F	0.033	***
Hydroxymatairesinol	0.24 ^A	0.16 ^{CD}	0.19 ^{BCD}	0.16 ^D	0.16 ^D	0.16 ^D	0.15 ^D	0.18 ^{BCD}	0.18 ^{BCD}	0.23 ^{AB}	0.23 ^A	0.21 ^{ABC}	0.020	**
Sum of plant lignans	0.79 ^{DEF}	0.72 ^F	1.05 ^{AB}	0.91 ^{CD}	1.11 ^A	1.06 ^A	1.02 ^{AB}	0.96 ^{BC}	0.78 ^{EF}	0.86 ^{CDE}	0.79 ^{DEF}	0.72 ^F	0.059	***
Mammalian lignans														
Enterolactone	54.4 ^{CDE}	58.1 ^{BCD}	80.6 ^A	78.5 ^A	75.0 ^A	65.7 ^B	59.8 ^{BC}	59.7 ^{BC}	55.7 ^{BCD}	54.9 ^{CDE}	49.2 ^{DE}	46.7 ^E	3.96	***
Enterodiol	0.32 ^{CDE}	0.28 ^{EF}	0.44 ^A	0.33 ^{DE}	0.38 ^{BCD}	0.37 ^{BCD}	0.40 ^{AB}	0.37 ^{BC}	0.29 ^{EF}	0.32 ^{CDE}	0.31 ^{CDE}	0.26 ^F	0.029	***
Sum of mammalian lignans	54.7 ^{CDE}	58.4 ^{BCD}	81.0 ^A	78.8 ^A	75.3 ^A	66.0 ^B	60.2 ^{BC}	60.1 ^{BC}	56.0 ^{BCD}	55.2 ^{CDE}	49.6 ^{DE}	47.0 ^E	3.978	***
Sum of lignans	55.5 ^{CDE}	59.2 ^{CD}	82.1 ^A	79.7 ^A	76.5 ^A	67.1 ^B	61.2 ^{BC}	61.0 ^{BC}	56.8 ^{BCD}	56.0 ^{CDE}	50.3 ^{DE}	47.7 ^E	3.994	***
Plant isoflavones														
Daidzein	1.84 ^{AB}	1.51 ^{ABC}	1.53 ^{ABC}	1.11 ^D	1.62 ^{ABC}	1.93 ^A	1.92 ^{AB}	2.04 ^A	1.72 ^{ABC}	1.61 ^{ABC}	1.52 ^{BCD}	1.45 ^{CD}	0.308	***
Genistein	1.95 ^A	1.47 ^B	1.01 ^{CD}	0.94 ^D	1.37 ^{BCD}	1.46 ^{BC}	1.48 ^{BC}	1.60 ^{AB}	1.71 ^{AB}	1.37 ^{BCD}	1.32 ^{BCD}	1.51 ^{BC}	0.283	***
Glycitein	2.55 ^A	2.41 ^{AB}	2.52 ^{ABC}	1.62 ^F	1.81 ^{EF}	2.25 ^{BCD}	2.09 ^{CDEF}	2.17 ^{BCDE}	2.01 ^{DEF}	2.03 ^{DEF}	2.24 ^{BCDE}	2.17 ^{BCDE}	0.194	***
Formononetin	0.65 ^A	0.47 ^{ABC}	0.29 ^{BC}	0.26 ^C	0.43 ^{ABC}	0.57 ^A	0.53 ^{ABC}	0.53 ^{AB}	0.64 ^A	0.49 ^{ABC}	0.49 ^{ABC}	0.51 ^{ABC}	0.182	*
Naringenin	0.35	0.25	0.25	0.20	0.25	0.22	0.21	0.19	0.18	0.19	0.21	0.20	0.032	†
Sum of plant isoflavones	7.33 ^A	6.11 ^{AB}	5.59 ^B	4.13 ^C	5.48 ^B	6.43 ^{AB}	6.22 ^{AB}	6.53 ^{AB}	6.25 ^B	5.70 ^B	5.78 ^B	5.85 ^B	0.863	***
Mammalian isoflavones														
Equol	252.0 ^{AB}	195.4 ^{BCD}	85.7 ^F	111.8 ^{EF}	103.4 ^{EF}	149.7 ^{DE}	183.3 ^{CD}	236.1 ^{ABC}	297.4 ^A	274.9 ^{AB}	264.8 ^{AB}	283.1 ^A	61.09	***
Sum of isoflavones	259.4 ^{AB}	201.5 ^{BCD}	91.3 ^F	115.9 ^{EF}	108.8 ^{EF}	156.1 ^{DE}	189.5 ^{CD}	242.7 ^{ABC}	303.7 ^A	280.6 ^{AB}	270.5 ^{AB}	288.9 ^A	61.88	***
Plant coumestants														
Coumestrol	0.26	0.22	0.35	0.18	0.23	0.17	0.18	0.22	0.26	0.27	0.23	0.25	0.069	ns

^a n=10, for each month^b ***, P < 0.001; **, P < 0.01; *, P < 0.05; †, 0.05 < P < 0.10 (trend); ns, P > 0.10. Different upper case letters within a row indicate significant differences between sampling month means (Fisher's Least Significant Difference test; P < 0.05)



—□— Conventional —△— Organic

Figure 1. Effect (P represents the ANOVA P-value) for the management system × month interaction on the concentrations of isoflavonoids in retail milk samples collected monthly over 12 months. Letters M to F, in Axis X, represent months between March 2016 and February 2017. Different upper case letters within a month indicate significant differences between means for the different management system within this month (Fisher's Least Significant Difference test; $P < 0.05$)



-□- Conventional -△- Organic

Figure 2. Effect (P represents the ANOVA P-value) for the management system × month interaction on the concentrations of lignans in retail milk samples collected monthly over 12 months. Letters M to F, in Axis X, represent months between March 2016 and February 2017. Different upper case letters within a month indicate significant differences between means for the different management system within this month (Fisher's Least Significant Difference test; $P < 0.05$)

APPENDIX

Table A1. Compound-Dependent LC-MS/MS Parameter, Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Cell Exit Potential (CEP).

Parameter assessed	Q1 mass (m/z)	Q3 mass (m/z)	DP (V)	EP (V)	CE (eV)	CEP (V)
¹³ C ₃ -enterolactone	300.0	191.9	-128	-10	-30	-14
¹³ C ₃ -enterodiol	304.1	255.1	-140	-10	-32	-17
Enterolactone	297.1	189.1	-140	-10	-26	-21
Enterodiol	301.1	253.1	-140	-10	-32	-19
Matairesinol	357.2	82.9	-145	-10	-26	-7
Hydroxymatairesinol	373.1	217.1	-115	-10	-32	-13
Secoisolariciresinol	361.2	165.0	-150	-10	-34	-11
Lariciresinol	359.1	329.0	-40	-10	-16	-21
Isolariciresinol	359.2	344.0	-165	-10	-26	-31
Pinoresinol	357.2	151.0	-155	-10	-24	-11
Syringaresinol	417.1	181.0	-170	-10	-26	-13
Medioresinol	387.2	151.0	-15	-10	-26	-25
Equol-d4	245.1	122.9	-75	-10	-20	-15
Daidzein-d3	256.0	225.9	-165	-10	-42	-13
Genistein-d4	273.1	135.1	-150	-10	-43	-11
Equol	241.1	119.0	-70	-10	-26	-8
Equol qualifier	241.1	134.9	-70	-10	-25	-8
Daidzein	253.0	223.0	-171	-10	-42	-15
Genistein	269.0	132.8	-160	-10	-42	-16
Glycitein	283.0	267.9	-75	-10	-25	-16
Naringenin	271.1	150.9	-121	-10	-25	-18
Formononetin	267.0	251.9	-147	-10	-28	-15
Chrysin	253.1	142.9	-128	-10	-30	-14
Coumestrol	267.0	211.0	-176	-10	-40	-11
Coumestrol qualifier	267.0	134.9	-176	-10	-39	-12

Table A2. Low Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ) and their Corresponding Regression Coefficients (*r*).

Parameter assessed	LLOQ Nm (ng/mL)	ULOQ Nm (ng/mL)	<i>r</i>
Equol	1.6 (0.39)	412.8 (100)	0.9958
Daidzein	0.038 (0.00977)	39.3 (10)	0.9991
Genistein	0.072 (0.0195)	37.0 (10)	0.9997
Glycitein	0.034 (0.00977)	35.2 (10)	0.9964
Naringenin	0.036 (0.00977)	36.6 (10)	0.9998
Formononetin	0.0089 (0.0024)	9.3 (2.5)	0.9998
Chrysin	0.049 (0.00195)	39.3 (10)	0.9996
Coumestrol	0.036 (0.00977)	37.2 (10)	0.9996

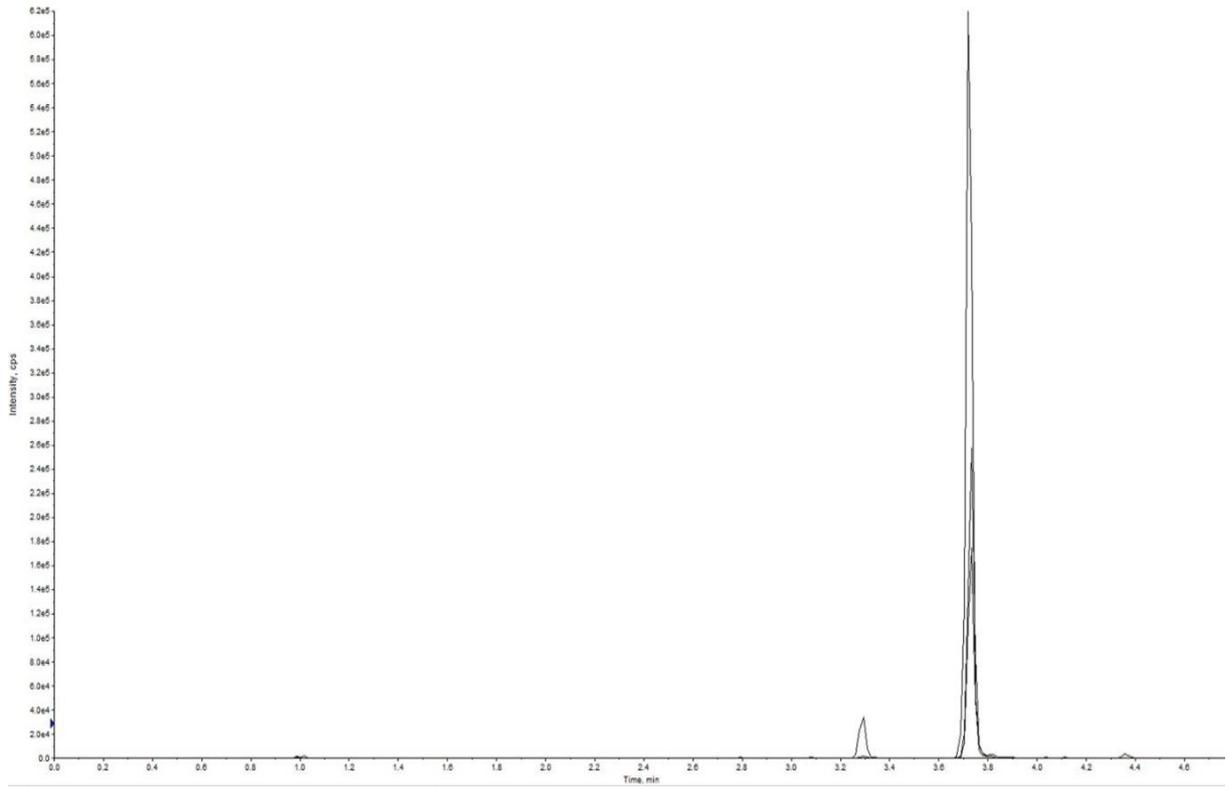


Figure A1. Total Ion Chromatogram of equol and internal standard equol-d4 in concentration of 100 ng/mL and 200 ng/mL respectively. Retention time of equol quantifier/qualifier 3.73 and equol-d4 3.72 min.

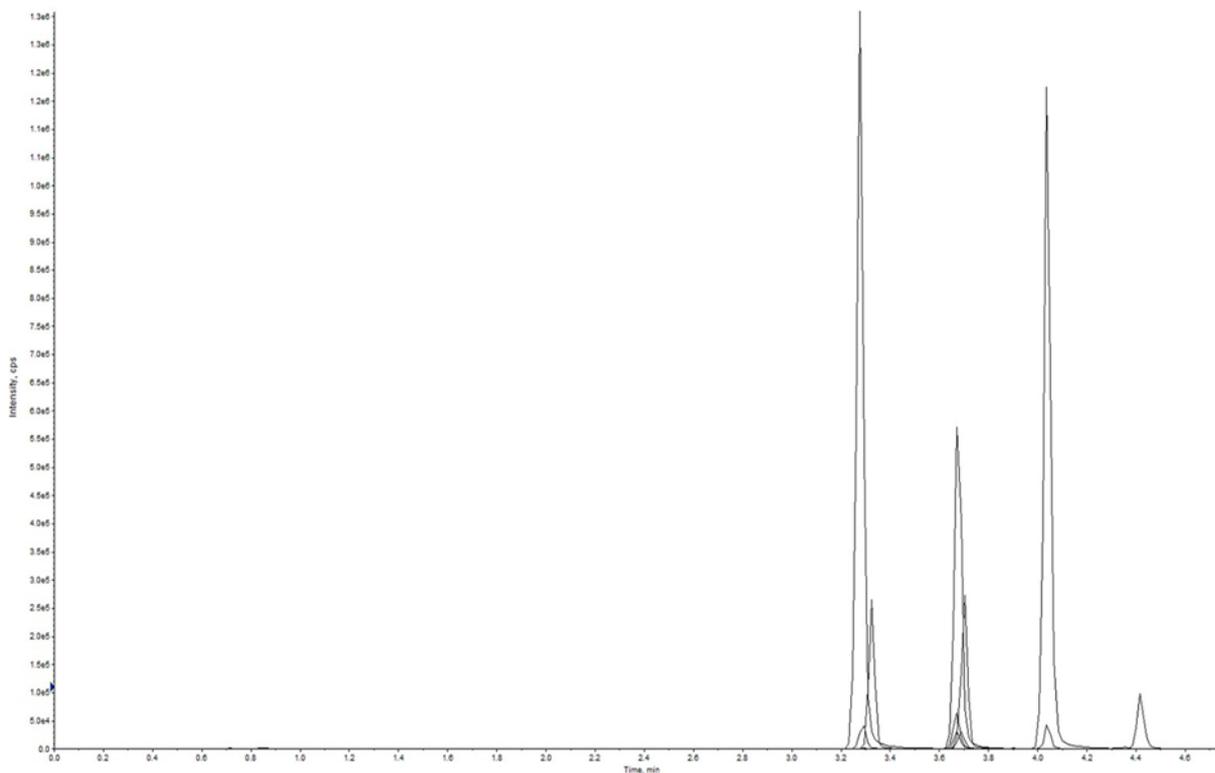


Figure A2. Total Ion Chromatogram of isoflavones standards in concentration of 1.25 ng/mL. The concentration of internal standards Daidzein-d3 and Genistein-d4 were 60 and 30 ng/mL respectively. Retention time in minutes for the standards were: Daidzein 3.28, Daidzein d4 3.28, Glycitein 3.32, Coumestrol quantifier/qualifier 3.67, Genistein 3.69, Genistein-d4 3.69, Naringenin 3.70, Formononetin 4.04 and Chrysin 4.42

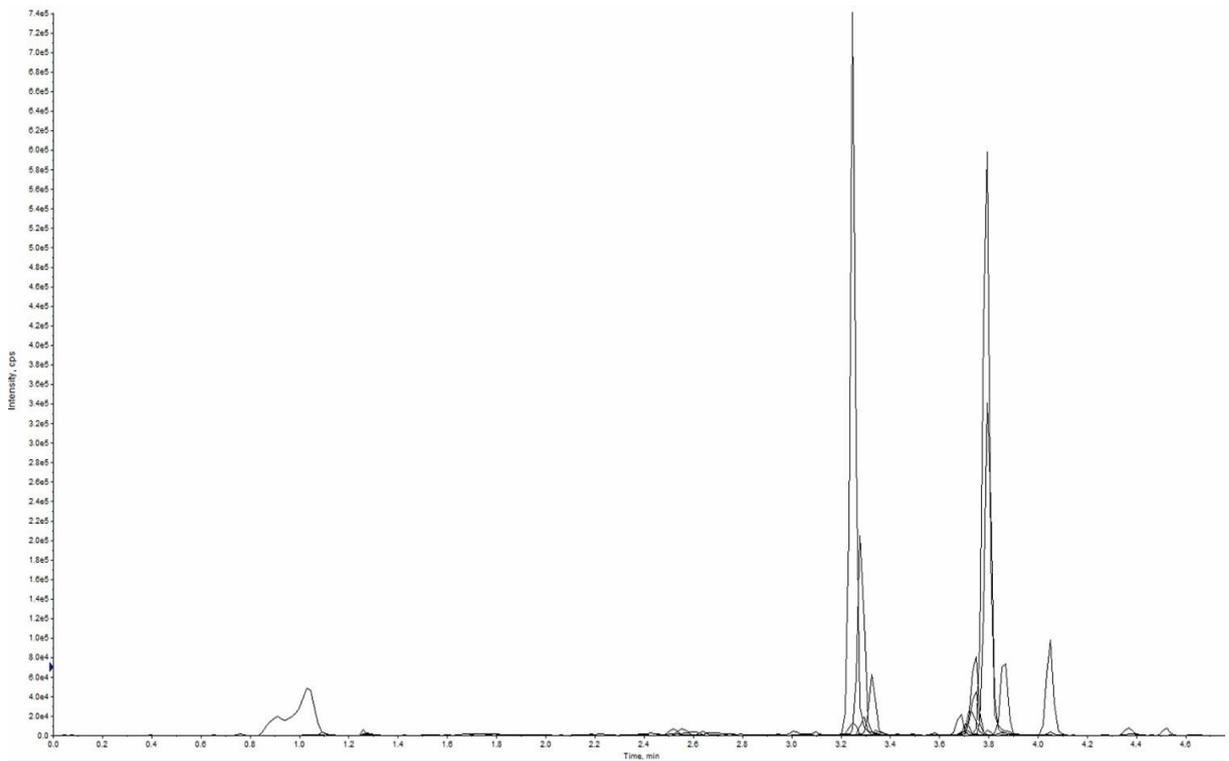
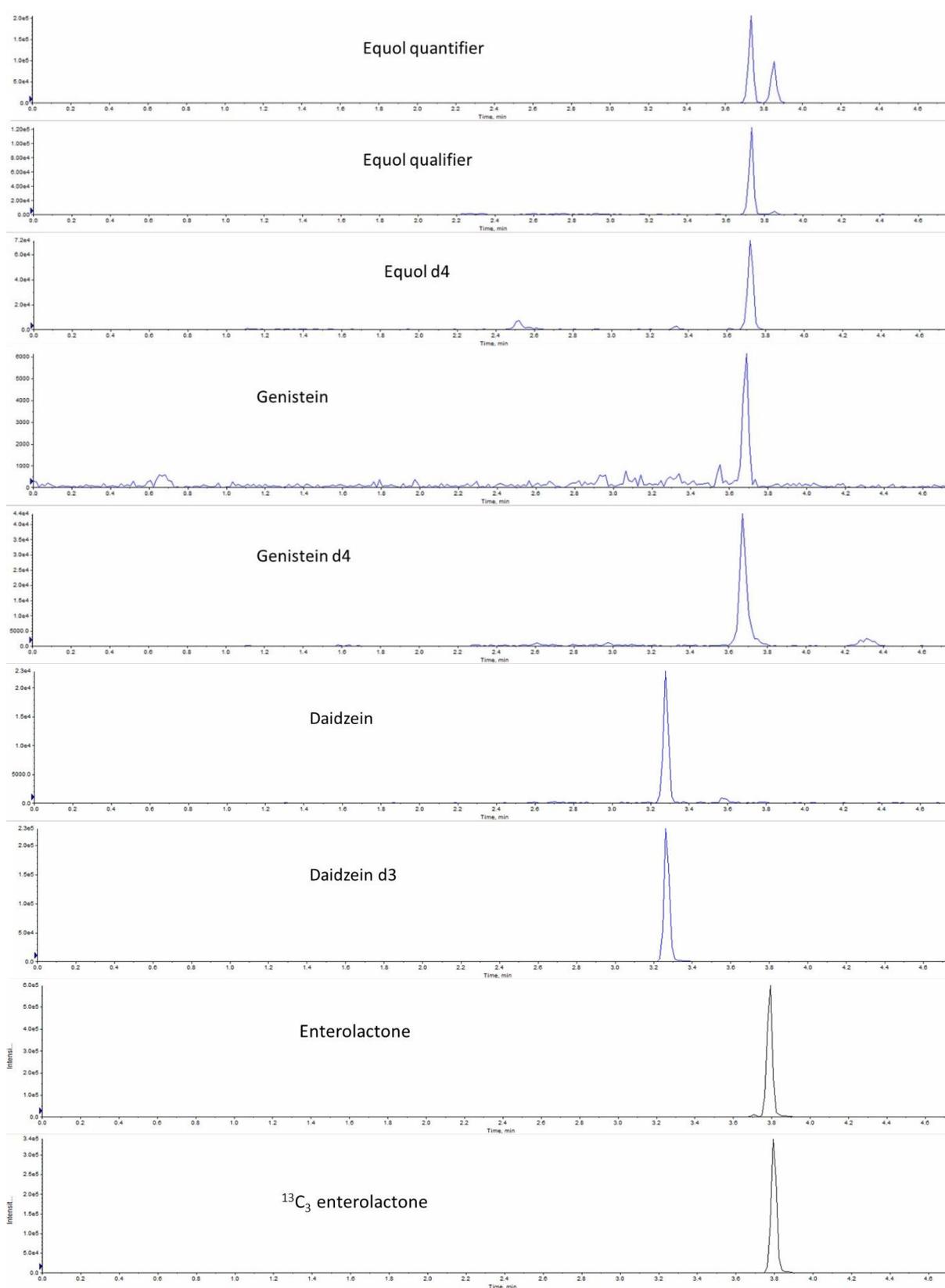
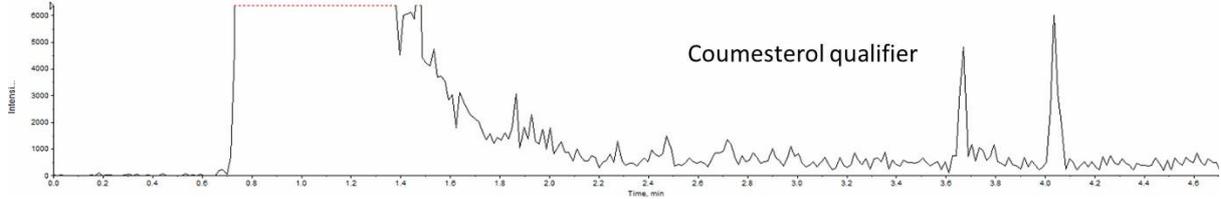
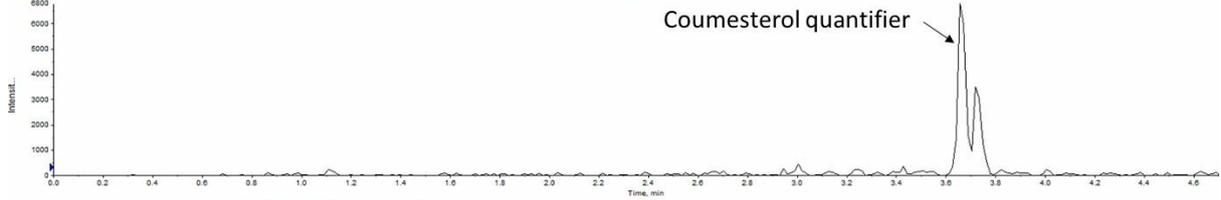
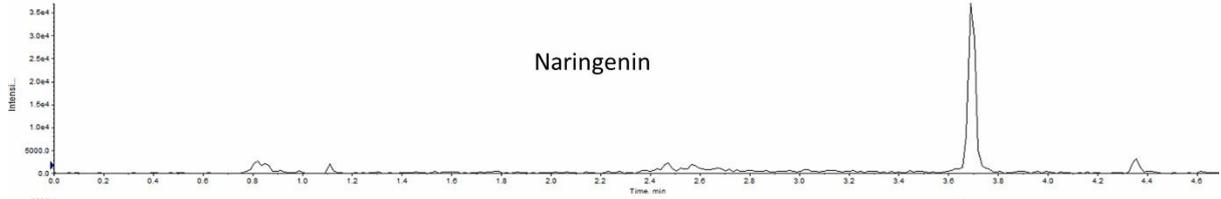
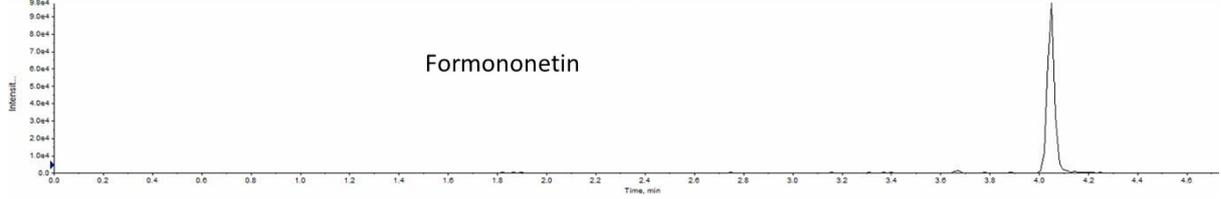
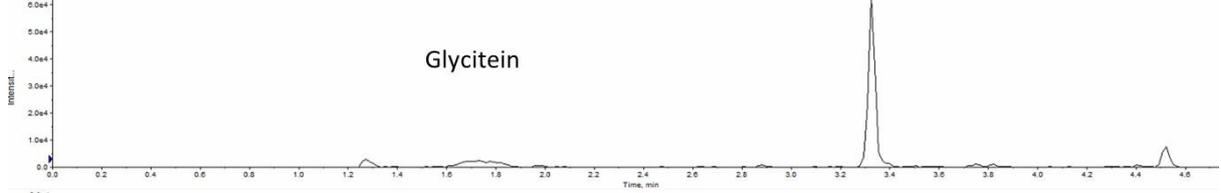
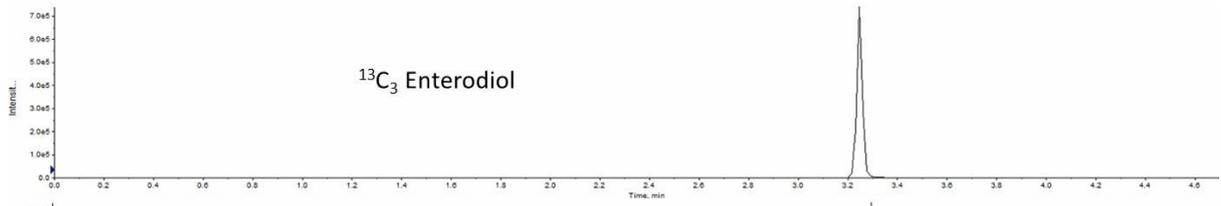
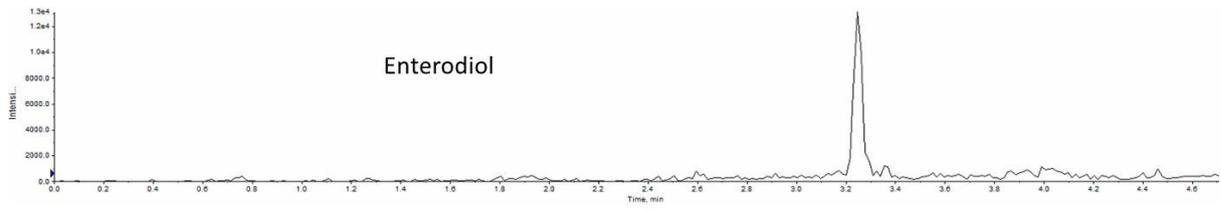


Figure A3. Total Ion Chromatogram of a milk sample for lignans and isoflavones and their corresponding internal standards.





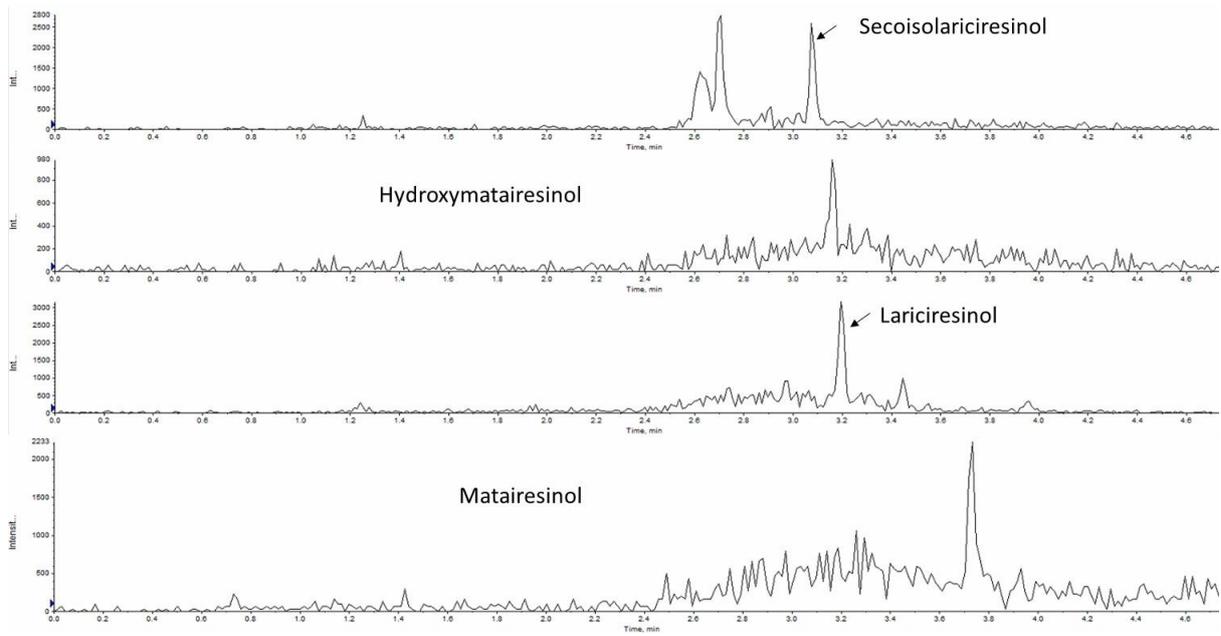


Figure A4. Extracted Ion Chromatograms of lignans and isovlavones and their corresponding internal standards in a milk sample.