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Suitability of bronopol preservative treated milk for fatty acid determination

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This work aimed to test if milk preserved with bronopol can be reliably used for fatty acid determination. Dairy production and milk quality are often monitored regularly to assess performance and contribute to selection indices. With evidence that fat composition can be influenced by selective breeding, there might be an interest in using samples collected in routine testing to evaluate individual cow fatty acid profiles, contributing to breeding indices. However, most recording services use a preservative such as bronopol and there is no published record if this influences subsequent fatty acid analysis. This study used milk from an oil seed supplementation trial, generating a wide range of milk fatty acid profiles, to test if the concentration of 31 individual fatty acids determined by GC were influenced by bronopol. Provided preserved samples are subsequently frozen, milk treated with bronopol can reliably be used to evaluate fatty acid composition in most cases; however bronopol might influence a few long-chain fatty acids present in relatively low concentrations. This is one small step towards simplifying milk compositional analysis but it could ultimately streamline the inclusion of milk fat quality into breeding indices, either with a view to 'healthier' milk or potentially reducing methane output and the environmental impact of dairy production.

Keywords: milk, fatty acids, preservative, bronopol.

Milk fat research has been taking place for over 100 years (Dewhurst, 2005), primarily focussed on manipulating fatty acid (FA) profiles to be less damaging or more beneficial to our health. More recent research suggests that fat composition can also potentially be used to predict methane output (Chilliard et al. 2009) and hence the environmental impact of milk production. In studies investigating the impact of management and feeding on fat quality using commercial farms (Ellis et al. 2006; Butler et al. 2008; Collomb et al. 2008; Rego, 2008; Slots et al. 2009) the standard practice prior to analysis is to store milk samples at − 20 °C until they accumulate, either over time or from distant locations. However, if we are to exploit genetic variation in fat composition (Lawless et al. 1999; Kelsey et al. 2003; Soyeurt et al. 2006) and selectively breed for greater desaturation of milk fat (Schennink et al. 2008), fatty acid profiles of individual animals need to be assessed.

Many dairy farmers carry out regular milk testing of individual cows under national recording services and it would be convenient if these samples could also be used for FA profiling in breeding programmes, focused on improving milk fat composition and environmental impacts (especially methane emissions of dairy production). Unfortunately, most milk recording services treat samples with a preservative such as potassium dichromate, azidiol or bronopol to eliminate the need for refrigeration or freezing the large volumes of samples. Due to the toxic and corrosive nature of potassium dichromate it is increasingly being substituted by the safer and more environmentally acceptable bronopol (2-bromo-2-nitro1,3 propanendiol) (Warne personal communication). The biocide action of bronopol is thought to relate to catalytic oxidation of thiols within microbial membranes and the generation of free radicals leading to cell death (Shepherd et al. 1988) which potentially could influence unsaturated FA in milk fat. There are limited studies demonstrating no significant effect of preservatives on total milk fat and protein content and/or somatic cell count (Bertrand, 1996; Monardes et al. 1996; Sanchez et al. 2005; Malinowski et al. 2008; Sierra et al. 2009) and one study reporting the impact of bronopol on mycotoxin content of sheep milk (Rubio et al. 2009). However, to our knowledge there are no published data on the effect of preservatives on FA, protein or antioxidants profiles in milk although some studies reporting fat composition have used bronopol preserved milk (Carroll et al. 2006; Collomb, 2008; Collomb et al. 2008).

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This study investigated possible handling protocols for FA determination if large numbers of milk samples are to be handled by the National Milk Recording services, UK (NMR), which involves collecting milk directly into tubes containing bronopol. It tests the null hypothesis that the method of preserving milk (mixing with bronopol and/or freezing) does not influence FA determination by gas chromatography (GC) when compared with the widely accepted procedure of freezing samples prior to analysis. A secondary study was also considered if the primary hypothesis was not upheld: if bronopol does influence fat composition, changes are consistent for all samples.

Materials and Methods

Sample collection and preservation

In order to test the hypothesis over a wide range of milk qualities, samples were collected from individual cows across treatments in an oilseed feeding trial, using 2 basal diets with and without supplementation (2 × 2 design). Milk from 4 cows in each of 4 dietary groups generated 16 milk samples with contrasting FA profiles, were used to investigate the influence of preservative treatment on fatty acid profiles assessed by a standard GC method.

Samples from afternoon and morning milkings for each cow were bulked within an hour of morning milking, mixed thoroughly and 3 sub-samples of 30 ml taken for this investigation. Two of the sub-samples were decanted into tubes containing bronopol solution (aiming for a final concentration of 20 mg bronopol per 100 ml milk) and mixed thoroughly (B); the 3rd sample remained untreated (U). Samples were then either frozen (F) at − 20 °C within 1 h of sub-sampling or kept at ambient room temperature (approximately 20 °C) for 24 h before being refrigerated at 4 °C for 8 days (A), mimicking transport from farms and subsequent refrigeration. No unfrozen samples without preservative were included since: a) ambient storage of untreated samples is not an acceptable practice in milk recording schemes, b) immediate GC analysis of milk samples from geographically dispersed commercial farms would not be feasible in practice and c) there are few or no studies reporting fatty acid results on previously unfrozen milk. Samples from the 3 treatments (UF – untreated and frozen, BF – bronopol treatment and frozen and BA- bronopol treatment and ambient/refrigerated) were then analysed.

Sample preparation and FA determination

Sample preparation and FA determination by GC was carried out by a variation of the widely used method of Sukhija & Palmquist (1988), as reported by Pickard et al. (2008), using methanol:toluene for lipid extraction and acetyl chloride for methylation of fatty acids prior to GC separation and quantification. If frozen, milk was thawed overnight at 6 °C and brought to room temperature. All samples were mixed thoroughly and a 0·5 ml aliquot transferred to a glass tube. 1·7 ml methanol:toluene (4:1 v/v) solution and 0·25 ml acetyl chloride were added before heating at 100 °C for 1 h in tightly sealed tubes. Samples were left for 30 min to reach room temperature before adding 5 ml potassium chloride. Samples were finally centrifuged at 1000 rpm for 6 min and the upper layer was removed for fatty acid analysis by GC.

Analysis of fatty acid methyl esters (FAME) was carried out with a GC (Shimadzu GC-2014, Kyoto, Japan) using a Varian CP-SIL 88 fused silica capillary column (100 m × 0·25 mm ID × 0·2μm film thickness). Purified helium was used as a carrier gas with a head pressure of 109·9 kPa and a column flow of 0·43 ml/min. The injection system (Shimadzu AOC-20i) used a split ratio of 89·8 and an injector temperature of 250 °C; detection by flame ionization detector was at 275 °C. Then 1μl of each sample was injected at an initial temperature of 50 °C, held constant for 1 min before being raised to 188 °C at 2 deg C/min, held for 10 min then raised to 240 °C at 2 deg C/min where the temperature was held for 44 min, giving a total runtime of 150 min. Peaks of individual fatty acids were identified using a 39 fatty acid FAME standard, composed of a 37 fatty acid standard (Supelco FAME mix C4-C24, 100 mg) and individual t11 C18:1 and c7c10c13c16c19 C22:5 standards, purchased by Sigma-Aldrich (Gillingham, UK). A separate standard with conjugated linoleic acid (C18:2 c9t11; CLA9) was kindly provided by colleagues from Danish Institute for Agricultural Science. Identification of peaks was confirmed by GC-MS (Shimadzu; GC-MS-QP2010; Kyoto, Japan) using the same column run under identical conditions. Peak areas for individual fatty acids were integrated using Shimadzu GC Solution software with quantification of individual fatty acids based on peak areas for each fatty acid as a proportion of total peak areas for all quantified acids.

Statistical analysis

The primary hypothesis was tested by general linear models ANOVA in Minitab (version 15) to compare fatty acid concentrations and some calculated values (total saturated, monounsaturated and polyunsaturated FAs, omega-3 and omega-6 FA) for individual cows and each of the 3 sub-samples using preservation method as the factor. Differences between preservation methods were assessed by Tukey's HSD tests. The secondary investigation used a two way ANOVA of FA results, this time using the dietary experimental group as the second factor, looking for potential interactions with preservation method.

Results and Discussion

As expected the divergent management and oilseed supplementation did generate milk samples with a wide range of fatty acid profiles; palmitic acid (C16:0) acid concentrations ranged from 230–379 g/kg total FA and oleic

Table 1. Mean milk fatty acid concentrations (g/kg total fatty acids) and *P* values from the Tukey Simultaneous Pairwise Comparisons Tests for 3 methods of preservation

	Mean value for each method†				ANOVA "Adjusted *P* value"		
	UF n=16	BF n=16	BA n=16	SEM‡	UF v BF	UF v BA	BF v BA
Individual fatty acids							
C4:0	25·4	24·9	23·8	0·5	0·87	0·47	0·23
C6:0	20·3	19·9	19·3	0·3	0·89	0·74	0·47
C8:0	13·1	13·1	12·9	0·3	0·99	0·95	0·90
C10:0	30·4	30·4	30·1	0·8	1·00	0·99	0·99
C11:0	0·87	0·78	0·52	0·07	0·86	0·33	0·14
C12:0	36·2	36·2	36·0	1·2	1·00	1·00	0·99
C13:0	0·85	0·82	0·83	0·03	0·90	1·00	0·94
C14:0	119	120	119	3	1·00	0·98	1·00
C14:1	9·29	9·14	9·26	0·48	0·99	0·99	1·00
C15:0	11·2	11·2	11·4	0·3	1·00	0·98	0·96
C16:0	311	311	311	6	1·00	1·00	1·00
C16:1	16·0	16·0	16·0	0·6	1·00	1·00	1·00
C17:0	6·12	6·25	6·38	0·14	0·93	0·92	0·73
C17:1	1·77	1·63	1·75	0·11	0·87	0·91	0·99
C18:0	124	123	124	4	0·99	1·00	1·00
C18:1 t11	10·1	10·2	10·2	0·5	1·00	1·00	1·00
C18:1 c9	216	216	218	6	1·00	0·99	0·99
C18:2 c9,12 *n*-6 (LA)	19·2	19·5	19·1	0·9	0·99	0·99	1·00
C20:0	2·28	2·28	2·56	0·19	1·00	0·80	0·81
C20:1	0·81	0·91	0·83	0·03	0·35	0·46	0·97
C18:3 c9,12,15 (αLA)	13·3	13·2	12·9	0·9	1·00	0·99	0·99
C18:2 c9 t11 (CLA9)	4·58	4·56	4·39	0·22	1·00	0·95	0·94
C22:0	1·83^{b}	1·93^{b}	2·73^{a}	0·11	0·88	**0·001**	**0·000**
C20:3 *n*-6	0·52	0·56	0·57	0·03	0·81	1·00	0·76
C20:3 *n*-3	0·70^{a}	0·58^{a}	0·31^{b}	0·05	0·47	**0·026**	**0·001**
C20:4 *n*-6	1·36^{b}	1·60^{b}	2·09^{a}	0·08	0·25	**0·006**	**0·000**
C22:2 *n*-6	0·43	0·59	0·60	0·04	0·23	1·00	0·21
C24:0	1·07^{b}	1·30^{a}	1·22^{ab}	0·04	**0·043**	0·65	0·25
C20:5 *n*-3 (EPA)	0·68	0·82	0·97	0·08	0·75	0·67	0·26
C24:1	0·22^{b}	0·40^{b}	0·72^{a}	0·05	*0·095*	**0·003**	**0·000**
C22:5 *n*-3 (DPA)	0·85	0·83	0·95	0·04	0·99	0·50	0·59
Calculated values							
SFA	704	703	701	8	1·00	0·99	0·99
MUFA	254	255	257	7	1·00	0·99	0·98
PUFA	41·6	42·2	41·9	1·7	0·99	1·00	0·99
n-3 PUFA§	15·5	15·4	15·2	0·8	1·00	0·99	0·99
n-6 PUFA¶	21·5	22·2	22·4	0·9	0·94	1·00	0·91
n-3:*n*-6 ratio	0·74	0·71	0·69	0·03	0·91	0·98	0·83

Key to abbreviations
[a] & [b] Means within a row with different superscripts differ ($P < 0·05$)
† Preservation methods: UF = No preservative and frozen, BF = Bronopol treatment and frozen, BA = Bronopol and stored ambient for 24 hours then refrigerated for 8 days
‡ SEM = standard error of the mean
§ Total *n*-3 FA: αLA (C18:3 c9 12 15), C20:3 c11 14 17, EPA (C20:5 c5 8 11 14 17) & DPA (C22:5 c7 10 13 16 19)
¶ Total *n*-6 FA: LA (C18:2 c9 12), C20:3 c8 11 14, C20:4 c5 8 11 14 & C22:2 c13 16

acid (C18:1 c9) between 155–343 g/kg total FA. Of the polyunsaturated FA concentrations; CLA9 ranged from 2·5–7·4 g/kg total FA and α linolenic acid (C18:3, c9, 12, 15) between 5·4 and 24·0 g/kg total FA. This variation is wider than average ranges suggested in a much cited review on the composition of bovine milk lipids (Jensen, 2002) since it reflects individual animal (rather than population) response to extremes in nutrition. The inclusion of both oilseeds and legume silage in dairy diets have been shown to increase milk unsaturated FA content at the expense of saturated FA (Kennelly, 1996; Dewhurst et al. 2002).

Results in Table 1 show that no significant effect of preservation treatments was identified for levels of: total saturated, monounsaturated, polyunsaturated, omega 3 (*n*-3)

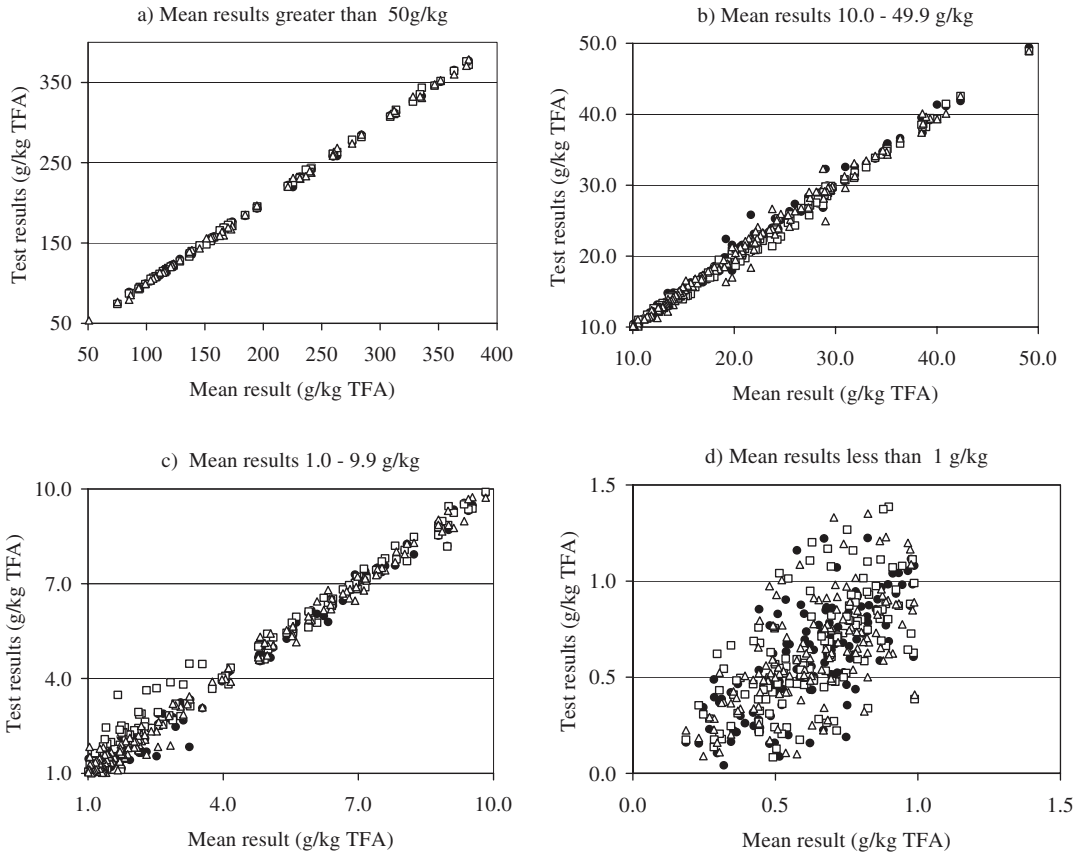

Fig. 1. Fatty acid concentrations from 3 methods of preservation relative to mean results (●=No preservative and frozen, Δ=Bronopol treatment and frozen and □=Bronopol treatment and ambient/refrigeration).

and omega 6 (*n*-6) fatty acids, the *n*-3:*n*-6 ratio and the vast majority of individual FA, together comprising 994 g/kg total FA. The use of bronopol to preserve milk in studies focused on these parameters is therefore unlikely to have a significant effect on FA composition. This is borne out in Fig. 1 which combines all results from the study, plotting individual fatty acid concentration for each of the 3 preservation treatment samples (y axis) against the mean of these 3 values (x axis), for all 31 individual fatty acids and 16 cows. These have been ranked (according to the mean FA concentrations for the 3 treatment results) and grouped to allow appropriate resolution to identify variation between treatment results. There is no obvious influence of storage treatment on FA concentrations for all results where the mean concentration is in excess of 4 g/kg total fat.

However, for some minor fatty acids (especially long-chain fatty acid), altogether amounting to approximately 6 g/kg total fat, significant effects of preservation methods were detected. When results from UF and BF samples were compared (Table 1), only one significant difference between treatments was identified for a minor saturated fatty acid, C24:0 with the bronopol treated samples having a higher concentration than the untreated sample (1·30 v 1·07 g/kg total FA, $P=0·04$). Comparing results from the ambient stored, bronopol treated samples (BA) with the untreated frozen (UF) milk, detected significant differences for a wider range of minor FA (C20:3 *n*-3, C20:4, C22:0 and C24:1) (Table 1), although together they accounted for less than 5 g/kg of total FA. When BF and BA samples were compared, significant differences were again identified for the same 4 minor FA, suggesting that freezing appeared to have a greater influence on fatty acid concentrations than the addition of bronopol (Fig. 1 & Table 1). The cluster of results for BA samples (□) above the mean line, towards the y axis in Fig. 1c, illustrates the higher concentration of C22:0 found in these samples relative to the UF and BF treatments (2·73 v 1·83 and 1·93 g/kg, $P=0·001$ and $P<0·001$ respectively).

Concentrations of C4 and C6 showed considerable variation (see outliers in Fig. 1b), however this appeared to be inconsistent with storage treatments (P values=0·23–0·89). The volatile nature of these short-chain FA is a more likely explanation and raises questions about the suitability of a relatively harsh analytical method to determine short-chain, volatile FA, which has been identified by other authors (Kramer & Zhou, 2001). Discrepancies in FA concentration were also identified among other minor fatty acids. With the exception of C20:3 *n*-3, C20:4 *n*-6, C22:0, C24:0 and C24:1 described above, the lack of significance for differences in these minor FA when comparing treatment means (Table 1) suggests this variation is due to inaccuracy and poor

repeatability in assessing their low concentrations rather than the preservation method. This is supported by the random nature to the scatter plot in Fig. 1d.

Since the biocide action of bronopol is thought to relate to the generation of free radicals (along with thiol oxidation; Shepherd et al. 1988) this could potentially influence the relative proportions of fatty acids measured since double bonds within the milk fat could be vulnerable along with those in microbial membranes. The presence of free radicals induced by bronopol might explain observed increases in the concentrations of long chain saturated; C22:0 and C24:0, (and possibly monounsaturated C24:1) FA and the depression in polyunsaturated FA: C20:3 and C20:4. However, why are these FA affected by bronopol whereas the relative proportions of other unsaturated or saturated FA appear unaltered? There are also inconsistencies between frozen and ambient bronopol samples when compared with the untreated, frozen milk. Of the 5 differences identified, most are only significant for the ambient stored bronopol samples (BA); freezing appears to block the impact of the preservative in this respect. On the other hand, results for C24:0 show the increase in concentration only reaches significance for the bronopol treated samples which were frozen.

Identifying these positive influences of preservation on some FA evoked the secondary study using this data. Closer inspection of results for this group of minor FA which were altered by preservation revealed that for 2 (C20:3 *n*-3 and C22:0), significant interactions also existed between these responses and the origins of the milk. Both interactions indicate inconsistency in the way preservative method impacts on milk fat but unfortunately, the nature of the interactions are very different making a potential explanation difficult. In the case of C22:0 the interaction appears to be to be driven by linseed supplementation, in contrast to that for the unsaturated FA C20:3 *n*-3 which is explained by response to the basal diet rather than supplementation. For both basal diets, cows fed linseed had higher concentrations of C22:0 in BA samples compared with UF and BF samples, whereas milk from cows receiving control diets showed no significant change in C22:0 due to preservation (interaction $P=0{\cdot}019$). On the other hand, the interaction identified for C20:3 *n*-3 ($P<0{\cdot}001$) stems from findings that milk derived from the two basal diets gave contrasting patterns of response to preservation method. Basal diet 1 produced milk with significantly lower concentrations of C20:3 *n*-3 in the BA treatment relative to other methods of storage whereas milk from cows on the other basal diet reacted very differently, with the UF samples showing elevated C20:3 *n*-3 compared with those treated with bronopol, although in the latter case this did not reach significance for cows on the linseed diet. This secondary study certainly indicated differences in how milk fat profiles react to freezing and/or addition of bronopol, dependant on the diet and supplementation of the cows. This might be explained by differing protection offered by antioxidants or other elements against free radicals deriving from the preservative but, in the absence of further information on milk quality or diet composition, it is difficult to understand or explain. However it does indicate an area warranting further investigation, using an experimental design targeted at shedding light on the reaction of these minor FA to oxidative challenge rather than the overall impact of preservation method on FA profiles as in this study.

Regarding the primary hypothesis, the main conclusion of this study would be that, provided (a) bronopol preserved samples are frozen soon after collection, and (b) the study does not focus specifically on long-chain fatty acids (20 carbon atoms or longer), milk samples preserved with bronopol can be reliably used to evaluate fatty acid composition by a standard GC method. Further work is needed to investigate any influence of preservatives on more sophisticated analytical methods such as individual CLA isomers or trans-fatty acids differentiation as well as the potential interactions with other components of milk that might influence the reaction of long chain fatty acids.

Whilst these results support the use of routine test samples preserved with bronopol to assess fatty acid profiles by GC as an initial step to identify cows with superior FA profiles, if we are to incorporate traits on milk fat quality such as *unsaturation* into breeding indices the next phase is a reliable, rapid, inexpensive method of FA determination in preservative treated milk, such as near or mid infrared spectrophotometry.

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