Preparation and characterization of control materials for the analysis of conjugated
linoleic acid and trans-vaccenic acid in beef

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Terry Engle, Amy S. Rasor, Nancy A. Conley

Abstract

Beef and beef fat control composites (CC) were prepared for a large multi-year study of conjugated
linoleic acid (CLA) and trans-vaccenic acid (TVA) in beef. Raw ground beef and beef suet were each frozen in liquid
nitrogen, ground to a fine powder, dispensed into subsamples, and stored at −60 °C under nitrogen. The CCs,
reference materials (beef/pork fat; meat homogenate), and standards prepared at two concentrations of CLA
and TVA added to a total lipid extract of beef (differing by 0.27 g/100 g CLA and 1.2432 g/100 g TVA) were
analyzed via alkaline saponification of lipids, derivatization to fatty acid methyl esters, and GC. CLA and TVA
recoveries from the standards were 95–113% (CLA) and 103–106% (TVA). Fatty acids quantified in the
reference materials were within the certified ranges. Relative standard deviations (n = 3 in each of 5 assay
batches) were ≤5% (TVA) and ≤10% (CLA) for both CCs, at both laboratories.

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1. Introduction

Conjugated linoleic acid (CLA) comprises isomers of linoleic acid (octadecadienoic acid, C18:2) with any combination of cis- or trans double bonds separated by a single bond. CLA containing a trans double bond may not have the same detrimental health effects as trans monounsaturated fatty acids (C18:1) found in partially hydrogenated oils (Banni & Martin, 1998; Chardigny et al., 2008; Park, 2009). Furthermore, CLA has potentially positive health effects (Rainer & Heiss, 2004). In animal models, the 9c11t isomer has been shown to have anticarcinogenic properties (Chin, Liu, Storkson, Ha, & Pariza, 1992; Ip, Chin, Scimera, & Pariza, 1991; Pariza, Park, & Cook, 2000). For example, rats fed CLA-enriched butterfat had a 50% reduction in the incidence of mammary cancer compared with rats fed butterfat with normal concentrations of CLA, following administration of a carcinogen (Ip et al., 1991). Based on other studies a number of other possible human health benefits may result from CLA, including prevention of atherosclerosis and reduced body fat (Pariza et al., 2000).

Trans-vaccenic acid (C18:1–11t; TVA) is a naturally occurring positional isomer of elaidic acid (C18:1–9t), the primary trans fatty acid in partially hydrogenated vegetable oils. There is evidence that TVA does not have the same negative cardiovascular effects as artificial trans fatty acids (Chardigny et al., 2008; Gebauer, Psota, & Kris-Etherton, 2007).

Both CLA and TVA are produced by microbial hydrogenation of unsaturated fatty acids in the rumen of ruminant animals (Banni & Martin, 1998). Therefore, cheese, butterfat, and meats are the main dietary sources of CLA and TVA (Chin et al., 1992; Schmid, Collomb, Sieber, & Bee, 2006). The primary CLA is the 9c11t isomer, although smaller amounts of 10r12c and others are also present in beef and...
dairy products (Cruz-Hernandez et al., 2006; Kramer et al., 2004; Pavan & Duckett, 2007). TVA is the main trans fatty acid in ruminant fats. Beef normally contains between 0.2 and 0.3 g total CLA per 100 g [calculated from Pavan & Duckett, 2007]) with higher levels of CLA occurring in products from grass fed animals (Leheska et al., 2008; Rule, Broughton, Shellito, & Maiorano, 2002). Attempts have also been made to increase the CLA content of meat by feeding animals particular types of feed or supplements to feed (Duckett, Andrae, & Owens, 2002; Migdal et al., 2004; Nuernberg et al., 2005). The TVA content of beef fat is typically between 2% and 5% of total fatty acids (Owens, 2002; Migdal et al., 2004; Nuernberg et al., 2005). The TVA containing products are typically meat fats. Furthermore, if acid hydrolysis is used, for example to extract total fat prior to derivatization of fatty acids to methyl esters (FAME) (Cruz-Hernandez et al., 2004; Kramer et al., 2004). Because of the complex nature of CLA and TVA analysis, matrix-specific food control samples and laboratory-specific method validation are especially critical for accurate quantitation and comparison of the CLA and TVA content of foods.

For a planned study of the CLA and TVA content of more than 40 different beef cuts, 6 composite samples from each of 36 to 72 animals will be assayed for CLA and TVA between 2008 and 2013. Method validation and a well-characterized control material are essential to validate the accuracy and precision of results for this set of samples across time and analytical runs in order to prevent differences from analytical variability being attributed to sample composition variability. Currently there are no commercially available meat or meat fat reference materials with certified CLA and/or TVA concentrations. A beef-pork fat blend (BCR CRM 163; Institute of Reference Materials and Methods, Geel, Belgium) has informational but not certified values for CLA. Standard mixtures of fatty acids and CLA are available from chemical suppliers, but, as mentioned, they cannot be used to assess the accuracy and precision of separation and quantitation of the analytes in the food matrix that must be extracted, saponified, and derivatized, separated and quantified in the context of many other fatty acids.

The goals of this study were to validate the analysis of CLA and TVA in beef fat, to prepare homogeneous control composites of beef muscle and beef fat, and to establish tolerance limits for CLA and TVA concentrations in the control composites using the validated methodology.

2. Materials and methods

2.1. Overview

To simulate quantitation within a beef fatty acid mixture, total lipid extracted from ground beef was spiked with accurate and precise amounts of CLA and TVA, at two concentrations. These spiked lipid extracts along with the standard solutions and unspiked lipid extracts were analyzed using the method to be validated, and recovery of
added CLA and TVA were calculated. Homogeneous composites of ground beef and of beef fat (suet) were prepared and dispensed among subsamples to be used as control composites in subsequent assays of beef samples. Each material was assayed in triplicate in each of 5 independent analytical batches, and preliminary tolerance limits were set for CLA and TVA concentrations.

2.2. Preparation of control composites

Ground beef (8.6 kg) labeled 25% fat was purchased locally in Blacksburg, VA for the beef control composite (beef CC). For the beef fat control composite (beef fat CC), beef fat (suet) (5.4 kg) was obtained from Jackson Frozen Food Locker (Jackson, MO).

The raw beef and raw beef fat were homogenized separately, to yield the two CCs. The suet was trimmed to remove bloody areas, muscle, and connective tissue. Both the beef and beef fat were cut into pieces of approximately 1 cm, frozen in liquid nitrogen, and homogenized to a fine powder in two (beef fat) to five (beef) batches, using a 6L stainless steel industrial food processor (Robot Coupe Blixer; Robot Coupe USA, Inc., Jackson, MS). For each CC, the batches were combined in a 30-qt stainless steel industrial food processor (Robot Coupe R30 Vertical Cutter-Mixer; Robot Coupe USA, Inc., Jackson, MS) while adding liquid nitrogen as necessary to keep the material frozen. The processor was pulsed for two sets of 5 s, then run for 30 s using the lowest speed setting. Subsamples of each CC were dispensed while frozen into subsamples of 10–12 g each in 30 mL glass jars with Teflon-lined lids (Qorpak catalog #GLC-07098; Qorpak, Bridgeville, PA), following standard methods developed in this laboratory (Phillips et al., 2006). The aliquots were sealed under residual nitrogen gas and stored at −60 °C. The subsample storage conditions were previously validated to maintain the stability of moisture and fat in homogenized food samples for at least 5 years (Phillips et al., 2001).

A total of 650 subsamples of the beef CC and 348 subsamples of the beef fat CC were generated. Each subsample was labeled with a unique identification code linked in a master sample tracking database to the sample description and information about preparation and distribution.

2.3. Preparation of quantitative CLA and TVA spiked beef lipid standards

Two levels of spiked beef lipid (12 aliquots each) with standard additions of CLA and TVA at low and high levels (which were approximately 50% and 100% of the expected endogenous concentrations), and 12 aliquots of unspiked beef lipid were prepared. A stock beef lipid solution was prepared by extracting total lipid from twelve portions of 1.2 g homogenized beef fat (~89% fat) using previously reported methodology (Phillips, Ruggio, & Amanna, 2008) and combining 50 mL (2 × 25 mL) precisely measured portions of each extract, by dispensing the solution into 50 mL glass test tubes using a Hamilton Microlab 900 (Fisher Scientific, Waltham, MA), to yield a total of ~8.0 g fat (12 × 1.2 g × 89% fat in the beef fat samples × 50 mL/80 mL = 8.0 g). The extracts were concentrated to 10–15 mL each under nitrogen at 40 °C, then quantitatively transferred with chloroform to one 200 mL volumetric flask and brought to volume with chloroform, yielding a total lipid concentration of ~40 mg/mL. Three 50 mL portions of this solution (each containing ~2.0 g fat) were accurately measured using separate 50 mL volumetric flasks. These three solutions were then quantitatively transferred to separate 200 mL volumetric flasks using chloroform to rinse. One of the three resulting beef lipid solutions was brought to volume (200 mL) with chloroform and mixed, to yield the unspiked beef lipid standard with a fat concentration of ~10.0 mg/mL. The remaining two 50 mL portions were spiked with CLA and TVA acid, one at the low level (~0.25 g CLA, 1.25 g TVA per 100 g fat and one at the high level (~0.5 g CLA, 2.5 g TVA per 100 g fat). The spiking was performed using a single stock standard solution (100 mL × −0.50 mg/mL CLA, −2.5 mg/mL TVA) prepared using CLA standard #05507 (a mixture of cis and trans 9,11- and 10,12-octadecadienoic acid, 99.4% purity) and TVA standard #V1131 (99.4% purity) obtained from Sigma-Aldrich Chemical Co. (St Louis, MO) added using Class A volumetric pipette (10 mL for low and 20 mL for high) into the 200 mL volumetric flasks containing the beef fat extract, before bringing to volume with chloroform and mixing. As an additional control, 20 mL of the spiking solution was also transferred to a 200 mL volumetric flask using Class A volumetric pipet, brought to volume with chloroform, and mixed well.

Each of the 4 final solutions was dispensed in precise 10 mL aliquots using the Hamilton Microlab 900 into 12 separate 16 mL amber glass vials with PTFE-lined caps, resulting in ~100 mg total lipid per vial.

All procedures were designed and performed with careful attention to preventing evaporation of the chloroform solvent. During the Hamilton Microlab operation (other than the initial fat extraction), Durafilm® (Diversified Biotech, Boston, MA) was wrapped around the top of flasks during dispensing to minimize evaporation. All other operations were performed quickly. After the final vials were dispensed the solvent was completely evaporated under nitrogen at −40 °C, caps were wrapped with Durafilm®, and the samples were stored upright at −60 °C.

2.4. Quality control of composite and standards preparation

2.4.1. Homogeneity of control composites

The homogeneity of the beef and beef fat CC was established by analysis of moisture in triplicate in each of six subsamples drawn across the dispensing sequence of each composite, as previously described for other control materials (Phillips et al., 2006).

2.4.2. Preliminary test of dispensing procedures for standards

The precision and accuracy of aliquots dispensed from the Hamilton Microlab has been well validated by tests with chloroform and in measurement of total lipid (Phillips et al., 2008). The procedure described above for preparing the beef lipid extract using volumetric flasks was tested using a solution of ~13 g sunflower oil added in a precisely weighed amount (13.4082 g) to a 1 L volumetric flask and taken to volume with chloroform. Sunflower oil was chosen to eliminate the need for lipid extraction. Twelve 25 mL portions of this solution were dispensed into separate 50 mL tubes, then dispensed as described above to a final set of three 200 mL volumetric flasks of oil solution with an expected concentration of 5.028 mg/mL. The contents of each flask was aliquotted in 10 mL portions using the Hamilton Microlab 900 as described above, with the 7th, 12th, and 17th subsamples dispensed from each flask being taken into preweighed dry 30 mL test tubes (12 total) for gravimetric determination of total lipid (Phillips et al., 2008). After evaporation of solvent under nitrogen, the mass of the lipid in each tube was compared to the expected amount, and these results were used to evaluate the accuracy and precision of the proposed dispensing procedure.

Additionally, the precision of dispensing the standard solutions using volumetric pipettes was validated. A C18:0 fatty acid methyl ester standard # N-18-M (Nu-check Prep Inc., Elysian, MN) (~99% purity) was selected as a recovery check based on the simplicity of its quantitation using standard fatty acid analysis, so that any variability in results would be related to inaccuracies in volumetric dispensing and not confounded by any variability in quantitation of CLA or TVA which are more difficult to precisely measure than C18:0. A solution of this C18:0 methyl ester standard was prepared at ~0.06 mg/mL and aliquotted in triplicate at 20 mL using 3 individual 20 mL Class A volumetric pipettes into 50 mL glass test tubes, evaporated to ~10–15 mL under nitrogen, quantitatively transferred into tubes containing C11:0 triglyceride internal standard, and quantified by GC-FID.

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using a previously published method (Phillips et al., in press), and precision was determined from these data.

2.4.3. Verification of transfer of lipid extracts from final vials

The ability to quantitatively recover the lipid from the vials prepared for analysis (e.g., after solvent has been evaporated and samples have been frozen at −60 °C) was assessed. The beef total lipid extract solution was aliquotted in triplicate immediately after preparation directly into 30 mL dry pre-weighed test tubes, and also recovered from 6 dispensed vials, three using hexane and three using chloroform for quantitative transfer. After overnight storage at −60 °C, the material in each vial was transferred to the pre-weighed tubes using the solvent (hexane or chloroform) (approximately 8 mL total) added in portions with vortex mixing and rinsing to achieve quantitative transfer. All tubes were then dried and total lipid was measured gravimetrically.

2.4.4. Validation of dispensing of beef lipid extract for final standards

During dispensing of the beef lipid extracts (unspiked, low spiked, and high spiked), 3 extra aliquots were taken (at the beginning, middle, and end of the aliquotting sequence) from among the 15 total vials used for each treatment for yielding a total of 9 subsamples. These aliquots were dispensed into pre-weighed dry 30 mL glass test tubes, and total lipid was gravimetrically determined as described above. The expected amount of lipid and the precision among aliquots were verified.

2.5. Validation of analytical methods

2.5.1. Analysis of CLA and TVA spiked beef lipid standards

Each of two prospective laboratories experienced in beef fatty acid analysis (Texas A&M University (TAMU), and Colorado State University (CSU)) received a sample set that included three aliquots each of the unspiked beef lipid, and beef lipid spiked with the low and high levels of CLA and TVA. The frozen vials were wrapped tightly in bubble wrap and shipped on dry ice via express overnight delivery to each laboratory. Laboratories were blinded to the sample identities, which were only indicated as “beef fat” or “beef”, and the replicates were specified to be run on separate days (i.e., completely independent runs of extraction and GC) to obtain an estimate of inter-assay precision. The laboratories were given the approximate mass of material and percent total fat in each vial and instructed to quantitatively assay CLA and TVA, to report fatty acids in g/100 g original sample and include the measured total lipid.

2.5.2. Analytical methods

2.5.2.1. TAMU. Total lipid was extracted by the method of Folch, Lees, and Sloane Stanley (1957), modified as described by Archibeque, Lunt, Gilbert, Tume, and Smith (2005). Fatty acid methyl esters (FAME) were prepared from saponified total lipid extracts as described by Morrison and Smith (1964) and analyzed with a Varian gas chromatograph (model CP-3800 fixed with a CP-8200 autosampler, Varian Inc., Walnut Creek, CA). Separation of FAME was accomplished on a fused silica capillary column CP-Sil88 [100 m × 0.25 mm (i.d.)] (Chrompack Inc., Middleburg, The Netherlands), with helium as the carrier gas (flow rate = 1.0 mL/min). Column oven temperature was increased from 150 to 225 °C and 1 °C/min, from 160 to 167 °C and 0.2 °C/min, from 167 to 225 °C and 1.5 °C/min, and then held at 225 °C for 16 min. The injector and detector were maintained at 250 °C. Total run time was 100 min. Individual FAME were quantified as a percentage of total FAME analyzed, and identified based on authentic standards.

2.5.2.2. CSU. Total lipid was extracted from 1 g of homogenized sample and from the standards, estimated to contain approximately 0.20 g total fat, using the method of Folch et al. (1957) as modified by Bligh and Dyer (1959). Saponification and methylation was accomplished using the method of Parks and Goins (1994). Briefly, lipids were extracted with chloroform:methanol (2:1, v/v), saponified by alkaline hydrolysis (0.5 N KOH in methanol) at 70 °C for 10 min. Fatty acids were derivatized to methyl esters by addition of 14% BF₃ in methanol and heating at 70 °C for 30 min, then reconstituted in hexane prior to GC analysis.

GC analysis was performed using a Hewlett Packard (Avondale, PA) Model 6890 series II gas chromatograph fixed with a series 7683 injector and flame ionization detector. The instrument was equipped with a 100-m × 0.25-mm (i.d.) fused silica capillary column (SP-2560 Supelco Inc. Bellefonte, PA). The carrier gas ramping temperatures and flow rate were the same as above. Fatty acids were quantified by incorporating internal standards (C12:0 and C27:0, 1 mg each) into each sample prior to methylation. C12:0 and C27:0 were not detected when standards were omitted from unknown samples. Fatty acid standards were obtained from Nu-Check Prep (Elysian, MN), Matreya, (Pleasant Gap, PA); and Supelco (Bellefonte, PA).

2.6. Characterization of the beef and beef fat control composites

Five subsamples of each CC were sent to TAMU and to CSU, and each subsample was analyzed at each laboratory in triplicate in each of 5 separate analytical runs using the validated methodology, and then each CC was sent again 2 months later and assayed in duplicate at each laboratory. The mean values and standard deviations were used to establish tolerance limits for total CLA and TVA concentrations in the CCs, according to standard principles for quality control of analytical measurements (Dux, 1990; Taylor, 1987).

2.7. Analysis of certified reference materials

Meat homogenate (NIST SRM® 1546) was obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD). BCR CRM 163 Beef/Pork Fat (Institute of Reference Materials and Methods, Geel, Belgium) was purchased from RT Corporation (Laramie, WY). Each reference material was analyzed in triplicate.

2.8. Data analysis

The assayed fatty acid concentrations are reported in g/100 g original sample, normalized to total fat as described by Weihrauch, Posati, Anderson, and Exler (1977), using 92% and 95% of total fat as the expected sum of fatty acids for the beef and beef fat samples, respectively. For NIST SRM 1546, the certified total fat concentration was the basis for normalization; for the control composites, the mean assayed total lipid concentration was used.

Means and standard deviations were calculated using Microsoft® Excel (Professional edition 2003; Microsoft Corporation, Redmond, WA). Analysis of variance (α = 0.05) was performed with Quattro Pro® (version 14.0.0.603; Corel Corporation, Ottawa, Ontario, Canada). Expected standard deviations were calculated from the assayed mean concentrations, and the HORRAT ratio (actual RSD/expected RSD) was calculated as described by Horwitz, Kamps, and Boyer (1980), using 0.67 times the expected inter-laboratory RSD as the intra-laboratory expected RSD; Z-scores for the assayed versus certified mean for certified fatty acids in the reference materials were calculated according to Jorhem, Engman, and Schröder (2001).

Recovery of TVA and CLA from the spiked beef lipid standards was calculated as the difference between the concentration measured in the spiked sample minus the mean assayed concentration in the unspiked samples, divided by the expected concentration based on the formulation of the spiked standards, corrected for the purity of the standards used.
3. Results and discussion

3.1. Homogeneity of control composites

The mean moisture content for the 6 aliquots taken throughout the dispensing sequence was 59.28 g/100 g with 0.36% RSD for the beef control composite, with no statistically significant difference between subsamples. For the beef fat control composite, a statistically significant lower moisture content (7.15 vs. 7.50 g/100 g) was determined in samples aliquotted from the last part of the sequence. Part of the reason for the statistical detection of the difference (which was < 0.5% and only 0.35 g/100 g) was the high precision of the assay. Nonetheless, the samples from the last part of the dispensing sequence were discarded, and the RSD across the final set of subsamples was 2.9%.

3.2. Quality control of preparation of spiked lipid standards

For the sunflower oil dispensed in the same manner as the beef lipid standards, the RSD for aliquots from within flasks was < 0.6% and the difference between the mean weight of lipid dispensed and the expected amount was the same for all three flasks [0.0001 g or 0.2%]. Overall, the RSD was 0.38% among the 12 aliquots. For the test of volumetric pipetting of the standard solutions, the amount of C18:0 fatty acid methyl ester added was 1.2178 mg. The three 20 mL portions dispensed yielded 1.2190, 1.2516, and 1.2089 mg, for a mean deviation of 0.71% from the expected amount, which is well within the error for the gravimetric measurement of total lipid.

3.2.1. Preliminary tests of preparation techniques

For the sunflower oil dispensed in the same manner as the beef lipid standards, the RSD for aliquots from within flasks was < 0.6% and the difference between the mean weight of lipid dispensed and the expected amount was the same for all three flasks [0.0001 g or 0.2%]. Overall, the RSD was 0.38% for the total of 12 aliquots.

For the test of volumetric pipetting of the standard solutions, 1.2178 mg of C18:0 FAME was added. The three 20 mL portions dispensed yielded 1.2190, 1.2516, and 1.2890 mg, for a mean deviation of 0.71% from the expected amount, which is well within the uncertainty of the gravimetric measurement of total lipid.

3.2.2. Validation of dispensing of beef lipid extract for standards

The RSD for the triplicate portions of the unspiked, low level and high level spiked standards taken through the dispensing sequence were all < 0.5%, and the mean gravimetrically determined total lipid per aliquot was 101.3 mg, 102.5 mg, and 104.0 mg, respectively, indicating good agreement with the expected amounts.

3.3. Validation of analytical methods

3.3.1. Recovery of TVA and CLA from spiked beef lipid extract

The recovery of TVA and CLA added at two concentrations to the beef lipid extract is illustrated in Fig. 1. The assayed concentrations

![Fig. 1. Recovery of trans-vaccenic acid (TVA) and conjugated linoleic acid (CLA) from beef total lipid (100 mg) spiked at two levels: low, 0.24705 mg CLA and 1.2432 mg TVA added; high, 0.4941 mg CLA and 2.4864 mg TVA added. Error bars represent 2 times the standard deviation of the assayed mean concentrations, and 2 times the expected standard deviation (Horwitz et al., 1980) for the expected concentrations.]

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Assayed Mean</th>
<th>%RSD</th>
<th>Assayed Mean</th>
<th>%RSD</th>
<th>Assayed Mean</th>
<th>%RSD</th>
<th>Difference</th>
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<tbody>
<tr>
<td>TAMU</td>
<td>1.038</td>
<td>7.8</td>
<td>1.3751</td>
<td>1.5004</td>
<td>1.5616</td>
<td>2.2</td>
<td>0.5175</td>
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<tr>
<td>CSU</td>
<td>1.2814</td>
<td>2.8</td>
<td>1.5313</td>
<td>1.5190</td>
<td>1.6430</td>
<td>0.7</td>
<td>0.0992</td>
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<tr>
<td>All</td>
<td>1.1926</td>
<td>9.6</td>
<td>1.5097</td>
<td>4.9</td>
<td>1.5851</td>
<td>4.0</td>
<td>0.0755</td>
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<tr>
<td>Trans-vaccenic acid</td>
<td>3.2182</td>
<td>0.6</td>
<td>4.5301</td>
<td>4.6022</td>
<td>5.8556</td>
<td>3.0</td>
<td>1.2601</td>
</tr>
<tr>
<td>TAMU</td>
<td>3.2260</td>
<td>2.2</td>
<td>4.6743</td>
<td>4.7335</td>
<td>5.8802</td>
<td>1.5</td>
<td>1.3341</td>
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<tr>
<td>CSU</td>
<td>3.4243</td>
<td>0.9</td>
<td>4.6508</td>
<td>4.8878</td>
<td>6.0470</td>
<td>0.3</td>
<td>1.2840</td>
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<tr>
<td>All</td>
<td>3.2212</td>
<td>3.5</td>
<td>4.6810</td>
<td>2.8</td>
<td>5.9650</td>
<td>2.1</td>
<td>1.3597</td>
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</table>

*Sample was lost.
Table 2
Assayed concentrations of certified fatty acids, conjugated linoleic acid (CLA), and trans-vaccenic acid in a commercial commercially available reference material (NIST SRM 1546, Meat Homogenate).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Certified concentration</th>
<th>CSU Mean</th>
<th>Range</th>
<th>%RSD</th>
<th>TAMU Mean</th>
<th>Range</th>
<th>%RSD</th>
<th>Z-scoreb</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.253 ± 0.02</td>
<td>0.229</td>
<td>0.217–0.235</td>
<td>4.3</td>
<td>0.230</td>
<td>0.229–0.230</td>
<td>0.3</td>
<td>−3.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>4.56 ± 0.39</td>
<td>4.618</td>
<td>4.605–4.640</td>
<td>0.4</td>
<td>4.535</td>
<td>4.523–4.551</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.17 ± 0.29</td>
<td>2.232</td>
<td>2.214–2.255</td>
<td>0.9</td>
<td>2.197</td>
<td>2.180–2.216</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.03 ± 0.01</td>
<td>0.031</td>
<td>0.028–0.033</td>
<td>10.0</td>
<td>0.030</td>
<td>0.027–0.032</td>
<td>9.9</td>
<td>−0.3</td>
</tr>
<tr>
<td>C18:1a</td>
<td>8.2 ± 0.96</td>
<td>8.237</td>
<td>8.211–8.278</td>
<td>0.3</td>
<td>8.332</td>
<td>8.307–8.378</td>
<td>0.4</td>
<td>0.3</td>
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<tr>
<td>C18:2a</td>
<td>1.96 ± 0.20</td>
<td>2.004</td>
<td>1.991–2.025</td>
<td>0.4</td>
<td>2.026</td>
<td>2.012–2.047</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>C20:4a</td>
<td>0.056 ± 0.025</td>
<td>0.084</td>
<td>0.077–0.096</td>
<td>12.8</td>
<td>0.086</td>
<td>0.084–0.092</td>
<td>5.7</td>
<td>14.0</td>
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<tr>
<td>C20:1a</td>
<td>0.156 ± 0.023</td>
<td>0.170</td>
<td>0.170–0.171</td>
<td>0.2</td>
<td>0.155</td>
<td>0.155–0.156</td>
<td>0.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

a National Institute of Standards and Technology (2008).
b Calculated according to Jorhem et al. (2001).
c Includes all measured isomers.

Table 3
Assayed concentrations of certified fatty acids, conjugated linoleic acid (CLA), and trans-vaccenic acid in BCR 163 Beef/Pork Fat certified reference material, assayed at two laboratories (CSU and TAMU), evaluated in the units given in the certificate of analysis (COA). FA = fatty acid; ME = methyl ester; RSD = relative standard deviation.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Assayed FA (g/100 g)</th>
<th>FA to ME Conversion Factor</th>
<th>Certificate of analysis</th>
<th>Assayed ME as % of total fatty acids</th>
<th>Assayed FA</th>
<th>Conversion Factor</th>
<th>Certificate of analysis</th>
<th>Assayed ME as % of total fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSU</td>
<td>TAMU</td>
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a Pocklington et al. (1993); COA indicates, “Includes, as appropriate, positional and geometrical (i.e. cis/trans) isomers”.
b Calculated according to Jorhem et al. (2001).
met the expected concentrations at both levels, from both laboratories. The average recovery by TAMU and CSU, respectively, were 100–113% and 94–101% for CLA and 105–106% and 103–106% for TVA. The assayed concentrations of TVA and CLA in the unspiked, low and high spiked beef lipid were significantly different ($p<0.15$), both within laboratory and for the total set of results from both laboratories, with acceptable precision for each beef lipid standard (Table 1).

It is worth noting that the expected 2 SD interval for the formulated (expected) concentrations of CLA in the low and high spiked beef lipid standards, which had a difference of 0.2705 mg/100 mg, overlapped, but the 2 SD intervals did not overlap for the assayed concentrations (Fig. 1). Since the expected SD is calculated from the analytical uncertainty expected at a particular nutrient concentration (being greater for nutrients at lower concentrations) (Horwitz et al., 1980), the results suggest that better than routine precision is necessary to detect this small difference in CLA concentration.

During routine analysis, peak identification was additionally confirmed by enriching selected samples with purified authentic standards of the specific fatty acids of interest and by increasing the typical GC run time to 120 min and using the temperature ramping profile as described by Duckett et al. (2002), which facilitated resolution and quantitation. These measures likely contributed to the demonstrated analytical and accuracy across assay batches.

### 3.3.2. Results for certified reference materials

Results for the certified fatty acids as well as the assayed TVA and CLA concentrations in NIST SRM 1546 meat homogenate and BCR 163 beef/pork fat are summarized in Tables 2 and 3. The Z-score was $<2$ and the RSD was $<5\%$ for all certified major (>0.5 g/100 g) fatty acids in NIST SRM 1546 and BCR 163, supporting the accuracy and precision of the results at both laboratories. The Z-score of 4.2 for C18:0 in BCR 163 from one laboratory might have resulted from variability in recovery of the fat for analysis, since the material is solid at room temperature, introducing an error for this material relative to the homogenized meat samples. This possibility was supported also by the relatively higher RSDs from this laboratory for the assayed concentrations of the major fatty acids in the beef/pork fat compared to NIST SRM 1546 meat homogenate (Table 2).

Only informational values are available in the certificate of analysis (COA) for CLA and C18:1 in BCR 163, and no values for these components are reported in the COA for NIST SRM 1546. The assayed concentration of total CLA in BCR 163 was considerably higher than the COA value (1.5 vs. 0.4 g/100 g in units of FAME as percent of total FAME, the units of certification for the RM). The assayed mean concentrations of the 9,11 isomer (0.33 and 0.25), which is the major CLA in beef, were closer to the informational value, suggesting that the concentration reported in the COA might include only this isomer.

The mean concentrations for total CLA and TVA in NIST SRM 1546 meat homogenate, combining results from both laboratories, were 0.020 g/100 g (8.8% RSD) and 0.049 g/100 g (12.4% RSD), respectively.

### 3.4. Characterization of the beef and beef fat control composites

Fig. 2 shows a representative chromatogram for the beef CC, and Fig. 3 summarizes the CLA and TVA concentrations assayed in the beef CC and beef fat CC. Good precision was achieved for both total CLA (RSD of 1.7%–9.6%) and TVA (RSD of 2.9%–8.0%) in both control composites assayed over six independent assay batches.

### 4. Conclusion

The tolerance limits established for CLA and TVA in the beef muscle and beef fat control composites, by analysis at two laboratories using analytical methodology that had been validated for accuracy using spiked beef lipid standards, allowed verification of assay performance in a subsequent study on the fatty acid composition of beef over 4 years (2008–2013), for which some data will be incorporated into the United States Department of Agriculture’s (USDA) National Nutrient Database for Standard Reference (USDA, 2009), beginning with Release 23 in 2010. Careful attention to quality control and method validation were critical for accurate quantitation, with precision adequate to discern meaningful differences in CLA and TVA concentrations among samples. Differences in TVA of 1.24 g/100 g lipid and 0.25 g total CLA/100 g lipid were achieved. Obviously the generalization of this power, determined from one set of spiked beef lipid standards, to all beef and beef fat samples is limited based on the variability in fatty acid composition that is possible. Any given study would need to establish the statistical power possible in that study, using specific validation of the accuracy and precision of analysis in the laboratory performing the analyses, and the methods presented could be used as a model. Any presentation of food composition data for these fatty acids without accompanying method

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**Fig. 2.** Representative chromatogram for the beef control composite. (1): C18:1–11t (trans-vaccenic acid); (2): C18:1–9c; (3): C18:1–11c; (4): C18:2–9c,12c; (5): C18:3–9c,12c,15c; (6): C18:2–9c,11t; (7): C18:2–11c,13t; (8): C18:2–10c,12t.
validation and quality control should be viewed with appropriate skepticism about its accuracy and precision.

Results for CLA and TVA in the commercially available meat homogenate and beef/pork fat reference materials should prove useful if other researchers include these commercially available reference materials in studies on meat and meat fat composition, so that the contribution of analytical differences can be separated from sample differences across studies.

Acknowledgments

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References


