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Improved GC/MS Method for Quantitation of *n*-Alkanes in Plant and Fecal Material†

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A gas chromatography−mass spectrometry (GC/MS) method for the quantitation of *n*-alkanes (carbon backbones ranging from 21 to 36 carbon atoms) in forage and fecal samples has been developed. Automated solid−liquid extraction using elevated temperature and pressure minimized extraction time to 30 min per sample as compared to more than 24 h for traditional GC−flame ionization detection methods that use saponification and liquid−liquid extraction. Extraction solvent requirements were also minimized to 10 mL per sample. Under optimal conditions, complete method recoveries, including extraction efficiency, were greater than 91%. The linear dynamic range was 5 to 100 nmol injected onto the column, with a limit of quantitation of 5 nmol. Intra-assay coefficients of variation for the analysis of annual ryegrass (*Lolium rigidum*), subterranean clover (*Trifolium subterranean*), and bovine feces ranged from 0.1%−12.9%, where lower concentrations of *n*-alkane produced a higher degree of imprecision. The reported GC/MS method permits simple, rapid, and precise quantitation of *n*-alkanes in plant and fecal material and reduces reagent and labor requirements.

KEYWORDS: *n*-Alkanes; dietary intake estimation; GC/MS

INTRODUCTION

Estimation of forage intake and dietary composition of grazing animals has been successfully accomplished using the naturally occurring fingerprint of *n*-alkanes present in the cuticular waxes of forage species (*1*−*4*). Comparing the *n*-alkane pattern in dietary plant materials to that acquired from the feces of an animal that has consumed the plants can be used to determine component species in the diet (*5*). The most widely used analytical method to quantatively measure the *n*-alkane content of forage and feces is that developed by Mayes et al. (*6*). This method involves potassium hydroxide (KOH) saponification of approximately 0.5 to 1.5 grams of material overnight at 90 °C, hot liquid−liquid extraction, and subsequent sample cleanup using silica gel column chromatography. The resulting eluent is then dried, redissolved in a known volume of either heptane or hexane, and analyzed by gas chromatography using a flame-ionization detector (GC/FID). Total analysis time is usually 16 to 18 h per sample set, and solvent requirements are estimated at 35 mL per sample. To improve the efficiency of this method in terms of reagent and labor requirements and reduce overall sample handling, an improved method was developed and validated. In the present article, the feasibility of using automated solid−liquid extraction at elevated temperature and pressure, on-line sample filtration, and detection using gas chromatography−mass spectrometry (GC/MS) to reduce sample handling, analysis time, and reagent requirements was investigated.

Table 1. Optimal Extraction Conditions for Automated Solid–liquid Extraction

ASE200 extraction parameters	
sample size	150 mg
extraction cell volume	1–mL
extraction solvent	Hexanes
temperature	100°C
pressure	1500 psi
preheat cell	5 min
heat cell after filled w/ solvent	5 min
static extraction cycle duration	5 min
flush volume (as % of cell volume)	10%
N_2 purge duration following extraction	60 s
# of extraction cycles	3

MATERIALS AND METHODS

Chemicals and Reagents. Anhydrous heptane (>99%), hexanes (HPLC grade), and sand (20−30 mesh) were purchased from Fisher Scientific (Pittsburgh, PA). Pure standards (>99%) for each *n*-alkane ranging in carbon chain length from 21 to 36 carbon atoms ($C_{21}H_{44}$ to $C_{36}H_{74}$; abbreviated as C_{21} to C_{36}) were purchased from Sigma-Aldrich (St. Louis, MO).

Assay. The developed and fully validated method for the quantitation of saturated hydrocarbons with chain lengths ranging from C_{21} to C_{36} reported herein involves an automated solid−liquid extraction and filtration process, followed by GC/MS analysis. Electron impact

† Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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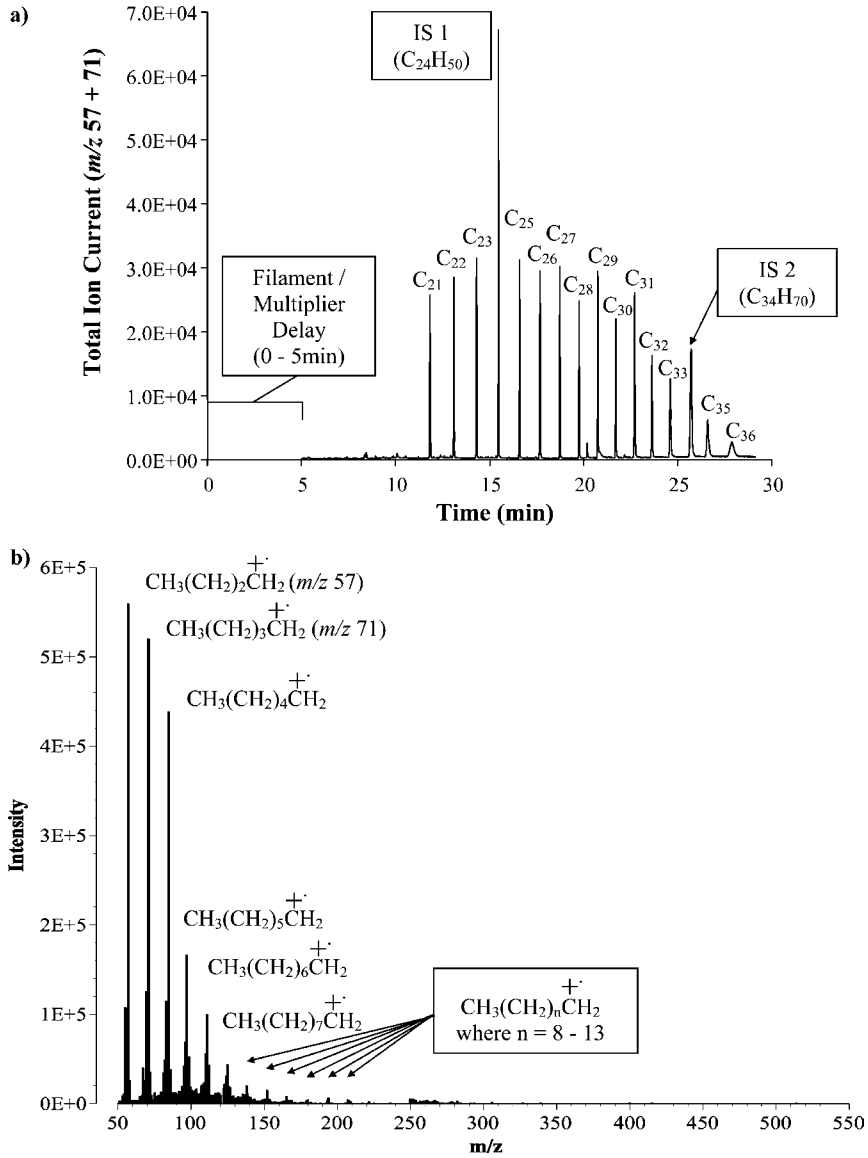

Figure 1. (**a**) Hay extract to which 12.5 nmol of each alkane with chain lengths ranging from 21 to 23, 25 to 33, and 35 to 36 carbon atoms were added (internal standard concentrations were 30 nmol), detected using single ion monitoring of *m*/*z* 57 and 71. (**b**) EI mass spectrum of $C_{29}H_{60}$ acquired scanning the full mass range from *m*/*z* 50 to 520.

ionization (EI) of the GC column eluents and subsequent single ion monitoring (SIM) on *m*/*z* 57 and 71, common fragment ions of *n*-alkanes detected in an ion trap mass spectrometer, provide a sensitive and precise analytical procedure.

Automated Solid−Liquid Extraction. An accelerated solvent extractor (ASE200; Dionex Corporation; Sunnyvale, CA) was used to automate the extraction and filtration process. The ASE200 accelerates traditional solvent extraction of solid samples by using solvents at elevated temperatures. Pressure is applied to the system to keep the heated solvent in a liquid state during extraction. The ASE200 first fills the extraction cell with solvent and pressurizes it at a user-defined setting. The cell body is then heated, beginning the first static extraction step, and the extraction solvent is held in the cell for a specified length of time. After this first extraction cycle is completed, a portion of the solvent, defined as a percentage of the cell volume, is flushed with nitrogen into a collection vial. If multiple extraction cycles are necessary, the cell is then refilled with solvent and pressurized. These extraction steps are repeated until the required number of cycles has been performed. Once all cycles are completed, the cell body is purged of solvent for a user-defined time interval with a stream of nitrogen.

Plant tissue was freeze-dried, and fecal material was dried in a forced-air oven at 55 °C for 48 h. Fecal samples were obtained from a beef steer consuming fescue hay and dosed with a slow-release bolus (Captec LTD., Auckland, NZ) designed to release approximately 317 mg of C_{32} and 335 mg of C_{36} per day. Samples were then ground to pass through a 1-mm mesh sieve. Samples were loaded into 1-mL stainless steel extraction cells designed for the ASE200 in the following order: First, a 6-mm cellulose filter disk was placed in the bottom of the cell. A layer of 20−30 mesh sand (~1 cm) was placed on top of the filter disk, followed by 150 mg of dried plant or fecal sample. Factors considered when the mass of ground sample to be extracted was determined included the density of the dried sample, the volume of the extraction cell body, and the anticipated quantity of alkanes present in the sample. A 100-μL aliquot of internal standard solution containing 3 mM $C_{24}H_{50}$ (Internal Standard #1) and 3 mM $C_{34}H_{70}$ (Internal Standard #2) was pipetted directly on the sample in the extraction cell. Finally, any remaining volume of the extraction cell was filled with sand to minimize the amount of solvent required to fill the cell during extraction. The ASE200 extraction parameters used for routine analyses of *n*-alkanes (C_{21}−C_{23}, C_{25}−C_{33}, C_{35} and C_{36}) from plant and fecal material are listed in **Table 1**. Extraction temperature and the number of extraction cycles to collect for a 150-mg sample size were optimized at 100 °C and 3 cycles, respectively. The final volume of the extract was ~6 mL, while the overall consumption of extraction solvent per sample was ~10 mL.

Following automated extraction, four 980-μL aliquots of extract were transferred to individual amber autosampler vials. A composite stock standard solution was prepared at a 1250 μM concentration level for each alkane in the standard solution, C_{21} to C_{23}, C_{25} to C_{33}, C_{35} and C_{36}. A total 20-μL aliquot was added to each sample vial to achieve the appropriate final concentration (i.e., 4 μL of 1250 μM standard solution and 16 μL heptane resulted in 5 nmol of each alkane added to the vial, 10 μL standard, and 10 μL heptane for a 12.5 nmol addition, and 20 μL standard for a 25 nmol addition). For the fourth 980-μL aliquot to which no alkane was added, a 20-μL aliquot of anhydrous heptane was added to bring the final total volume to 1000 μL, as in the other vials, and similarly diluted any potential matrix components. The sample set was stored at 4 °C until analysis by GC/MS, usually no longer than overnight following the addition of the standard solution.

Gas Chromatography−Mass Spectrometry (GC/MS). Capillary gas chromatography, specifically a model 3800 system coupled to a Saturn 2000 ion trap mass spectrometer (Varian, Inc.; Walnut Creek, CA), was used for method development and validation. The capillary injection port contained a deactivated gooseneck (tapered) inlet liner, inserted with the tapered end toward the head of the capillary GC column. The inlet was operated in splitless injection mode at 300 °C and was purged for 0.75 min following the injection of 1 μL of sample extract. The split vent on the injector port was programmed to then open at a split ratio of 1:100. The autosampler needle was heated in the injection port for 10 s after injection to aid the vaporization of longer chain alkanes and minimize peak tailing for C_{30} and longer carbon chain lengths. A pressure pulse upon sample injection of 70 psi for 0.75 min was used.

A GC capillary column with a low bleed arylene stabilized stationary phase equivalent to 5% phenyl/95% dimethylpolysiloxane (VF5-ms; 30 m × 0.25 mm ID × 0.25 μm film thickness; Varian, Inc.; Walnut Creek, CA) was used to optimize the separation of the analytes. Helium was the carrier gas applied at constant flow of 1.5 mL/min. The column oven was temperature programmed from 135 to 325 °C at 7 °C/min, held at 325 °C for 2 min, ramped to 350 °C at 20 °C/min, and held at 350 °C for 4 min. The ion trap, manifold, and transfer line of the mass spectrometer were held at 200, 80, and 180 °C, respectively. The filament emission current for the ionization source was 10 μamps, and the electron multiplier was set at 1300 V. The source filament and electron multiplier remained off during the initial 5 min of the GC column oven temperature program to allow the unretained solvent front to elute without detection. The mass range scanned was 50 to 520 amu at 587.5 amu/s for the initial confirmation of alkane identity and peak purity. For routine analyses, the mass analyzer was set for single ion monitoring on *n*-alkane fragment ions $CH_3(CH_2)_2CH_2^{+\cdot}$ and $CH_3(CH_2)_3CH_2^{+\cdot}$, detected with mass-to-charge ratios (*m/z*) of 57 and 71, respectively.

Data Analysis. Alkanes C_{21}−C_{23} and C_{25}−C_{27} were quantitated using C_{24} as an internal standard, while better accuracy and precision were obtained for C_{28}−C_{33}, C_{35}, and C_{36} when C_{34} was used as a second internal standard. Standard addition calibration curves were generated by plotting the ratio of peak areas for a given alkane to the internal standard (C_n/C_{24}, where n = 21−23 and 25−27; C_n/C_{34}, where n = 28−33, 35, and 36) against the concentration ratio of alkane added to the original sample to the corresponding internal standard. The absolute value of the *x*-intercept of an extrapolated best-fit linear trendline, multiplied by the internal standard concentration, was equal to the unknown alkane concentration in the original sample.

Matrix Effects. To assess the degree of ion suppression that might be caused by matrix components present in the extract, the slopes of the best-fit linear trendlines for calibration curves of pure *n*-alkane working standards diluted with anhydrous heptane without matrix interferences were compared with the best-fit linear trendline slopes for samples in which *n*-alkanes were spiked in the sample extract. Calibration curves were generated for a series of working standards prepared by serial dilution of a stock standard mixture of alkanes (~1000 μM for each of C_{21}−C_{23}, C_{25}−C_{33}, C_{35}, and C_{36}). The internal standards, C_{24} and C_{34}, were spiked into every working standard solution at a final concentration of 30 μM. Samples of annual ryegrass (*Lolium rigidum*), subterranean clover (*Trifolium subterraneum*), and bovine fecal matter were extracted as previously described. Calibration curves were produced from these standard addition samples for comparison with the calibration curves made from the series of working standards using fragment ions from the mass spectra as previously described.

Complete Method Recovery. Three 1-mL ASE extraction cells were loaded with a filter disk and filled with sand. A 25-μL aliquot of 1250 μM alkane stock standard solution was spiked into each cell to achieve a final concentration of 31.25 μM (in 1 mL of extract), assuming complete method recovery. Each extract was analyzed by GC/MS as described in triplicate. The overall efficiency of the method, including extraction efficiency, was calculated as the percentage of alkane recovered with respect to the initial spiked concentration. The recovered alkane concentration was determined by interpolation of peak area ratios (alkane to corresponding internal standard) on a calibration curve produced from serially diluted alkane stock standard solution in heptane.

RESULTS AND DISCUSSION

System Suitability and Specificity. To determine the suitability of the GC/MS system for quantitative analysis of saturated hydrocarbons, a variety of tests were imposed on data acquired using the optimized method parameters described in the Materials and Methods section.

System suitability tests are an integral part of chromatographic methods and are used to verify that the resolution and reproducibility of the system are adequate for the analysis. The three factors typically measured and reported are peak resolution (how well an analyte is separated from other mixture components), the number of theoretical plates (related to GC column efficiency), and peak tailing factors. The results for the GC/MS system on which this method was developed and validated were calculated from data acquired for the *n*-alkane content of hay material to which 12.5 nmol of each alkane, C_{21}−C_{23}, C_{25}−C_{33}, C_{35}, and C_{36}, was added (**Figure 1a**). Peak resolution (R) for closely eluting *n*-alkane and matrix peaks was calculated using eq 1 in which R is the resolution, t_{R2} and t_{R1} are the retention times of the second and first eluting peaks in seconds, respectively, and w_2 and w_1 are the peak widths in seconds measured at 10% peak height for the second and first eluting peaks, respectively.

$$R = [(t_{R2} - t_{R1})/0.5(w_1 + w_2)] \quad (1)$$

For reliable quantitation, *n*-alkane peaks must be well separated from any other eluting mixture component. The greater the calculated *R*-value for peak resolution, the better the eluting peaks are separated. Commonly, an acceptable limit for R is greater than or equal to 2.0, indicating that the analyte peak of interest elutes at a retention time that is different from the closest potential interfering peak by twice the average peak width (*7*). Interfering peaks may be due to the presence of an impurity from internal standard added to the sample extract, a degradation product of the internal standard, the internal standard itself if peak resolution is poor, extraction solvent impurities, or other compounds extracted from the original sample. Typical peak resolution (R) ranged from 2.0 to 2.8 using the developed GC/MS method. The original *n*-alkane GC/FID method developed by Mayes et al. in 1986 required sample cleanup with silica gel column chromatography. The developed method reported using automated solvent extraction-provided baseline peak resolution for all alkane chain lengths (**Figure 1a**), indicating that silica gel cleanup was unnecessary to achieve well-separated peaks in the chromatogram. Omitting this cleanup step decreased the overall required sample preparation time and the amount of sample handling, eliminating an opportunity for analyte loss.

The number of theoretical plates (N), a measure of column efficiency, for the VF-5ms capillary column used to validate this method was calculated using eq 2, where t_R is the retention

time for an eluting peak in seconds, and w is the width of that peak at 10% peak height in seconds.

$$N = (16 \times t_r^2/w^2) \quad (2)$$

Generally, N should be greater than or equal to 2000, indicating that the chosen capillary column is of sufficient length and efficiency to achieve good separation for the investigated *n*-alkanes (*7*). The number of theoretical plates ranged from 8.4×10^3 to 2.1×10^5 and was well above this limit. In fact a shorter capillary column could be used, resulting in earlier retention times; however, peak resolution would be adversely affected by doing so.

The final measure of system suitability, peak tailing factor (TF), was calculated for each *n*-alkane peak using eq 3, where w_{total} is the width of the peak at 10% peak height in seconds, and w_{front} is the width of the front half of the peak, also measured at 10% peak height, in seconds.

$$TF = (w_{total}/2w_{front}) \quad (3)$$

The degree of peak tailing varied for early and late eluting alkanes, causing the tailing factor to range from 1.04 to 1.15. A tailing factor of 1.0 indicates that the chromatographic peak is perfectly symmetric, while a generally acceptable range for TF is 0.5 to 2.0 (*7*). As a result of the mediocre quality of preliminary data (not shown) in terms of unacceptable degrees of peak tailing (typically, TF > 4) and mass discrimination (unequal detector responses for equimolar injections of *n*-alkanes) for longer chain alkanes ($>C_{29}$), several parameters had to be optimized. Initial attempts to analyze *n*-alkanes with a straight-through injection inlet liner resulted in half the overall signal relative to that acquired using a gooseneck liner. Additionally, inverting the gooseneck liner in the injection port so that the head of the GC column was directly inserted in the tapered end of the liner successfully trapped the injected sample aliquot temporarily. This orientation of the liner allowed a longer time frame for more complete vaporization of *n*-alkanes with longer chain lengths and thus higher boiling points. Originally, a constant flow of 1.5 mL/min (~11psi at 150 °C) through the inlet during injection was used. Applying a pressure pulse during sample injection in which the inlet pressure is increased immediately prior to injection and returned to the normal analytical set value after a specified amount of time has been reported to reduce sample discrimination (*8*). Incorporating a pressure pulse of 70 psi for 0.75 min upon injection helped further reduce mass discrimination for long chain alkanes and increased the overall signal for all alkanes analyzed approximately 5-fold (data not shown).

Specificity. The mass spectrum for $C_{29}H_{60}$ acquired with electron impact ionization (EI) following GC separation of a hay extract is shown in **Figure 1b** and is representative of the mass spectra for all alkanes included in this study. Upon ionization, enough energy was deposited into the eluting alkane that extensive fragmentation occurred for all chain lengths, and no molecular ion was observed. However, the resulting fragmentation pattern of sequential losses of 14 Da (equivalent to loss of a methylene group) is characteristic of saturated hydrocarbons. Furthermore, the mass spectrum for each eluting alkane provides evidence that the detected response at each retention time is pure and not due to coelution with non-alkane matrix components. Chemical ionization (CI) with acetonitrile is another method by which eluting *n*-alkanes can be ionized prior to mass analysis. Previous literature has reported this ionization method as a means to ionize long chain alkanes for which less fragmentation is observed, and the molecular ion may be used for quantitation purposes (*9*). The main advantage for using an ion with a higher mass-to-charge ratio (m/z) for quantitation is a lower probability that a matrix component will also form an ion with the same m/z value. CI was attempted in the early stages of method development using acetonitrile as a reagent gas, and while larger ions were observed (e.g., molecular ion adducts with reagent gas $(M+CH_3CN)^{+\bullet}$), the overall signal was much lower, and the overall sensitivity of the method suffered. Because there were no matrix ions in the samples investigated that prevented the use of EI due to coelution, it was chosen for the greater sensitivity it provided.

Linearity and Range. The linear dynamic range of this method was determined by plotting calibration curves for a series of working standards prepared by serial dilution of a stock *n*-alkanes standard mixture (~1000 μM for each alkane, $C_{21}-C_{23}$, $C_{25}-C_{33}$, C_{35}, and C_{36}). The chosen internal standards, C_{24} and C_{34}, were spiked into every working standard solution at a final concentration of 30 μM. The lower limit of the linear dynamic range, also known as the limit of detection (LOD), was defined as the concentration at which a signal-to-noise ratio of 3 is routinely obtained (*10*). For this assay, an alkane concentration of 2.5 μM (equivalent to 5 pmol injected on the column) is the LOD for all chain lengths. The upper limit of the linear dynamic range was determined on the basis of GC capillary column capacity, not MS detector response. The plateau of MS detector response with increasing concentration was never reached and was thought to occur at a concentration much greater than the expected concentrations of *n*-alkanes extracted from plant and fecal samples. The typical range of linearity was determined to be 5 to 50 nmol as shown in **Figure 2** as open squares. At quantities greater than 50 nmol, chromatographic peaks were asymmetric and exhibited peak shapes indicative of column overloading (i.e., peak fronting). The limit of quantitation, LOQ, was defined as the concentration at which a signal-to-noise ratio of 10 is routinely obtained (*10*). For this assay, an alkane concentration of 5 μM (equivalent to 10 pmol injected on the column) is the LOQ for all chain lengths.

To understand the role that matrix components may play in ion suppression, a range of alkane standards was prepared using extraction aliquots rather than pure anhydrous heptane as the diluent and analyzed using the developed GC/MS method. The slopes of the standard addition curves for C_{22} and C_{30} were compared to the trendline slopes for pure working standards. These alkane chain lengths were chosen for comparison on the basis of the apparent absence of these alkanes in the plant and fecal extracts (*1*, *11*). The concentration of these alkanes added to the extract should not be significantly increased by any extracted alkane from the sample. As shown in **Figure 2a** and **b**, the degree of ion suppression varied depending upon alkane carbon chain length and concentration. Shorter chain lengths from C_{21} to C_{27}, represented by C_{22} (with respect to C_{24} as the internal standard) in **Figure 2a**, were suppressed only slightly by matrix ions as indicated by a greater slope for the working standards without matrix. However, C_{28} and longer alkanes showed gradually lower signals for increasing alkane concentrations (i.e., the trendline slope was lower for the extract matrix than the working standards). This is represented by C_{30} (with respect to C_{34} as the internal standard) in **Figure 2b**. Even when C_{24} was used as the internal standard for C_{30}, matrix effects were still observed by the lower slopes for the extract samples (dashed line in **Figure 2b**). The difference in trendline slopes when C_{34} was used as the internal standard and when C_{24} was

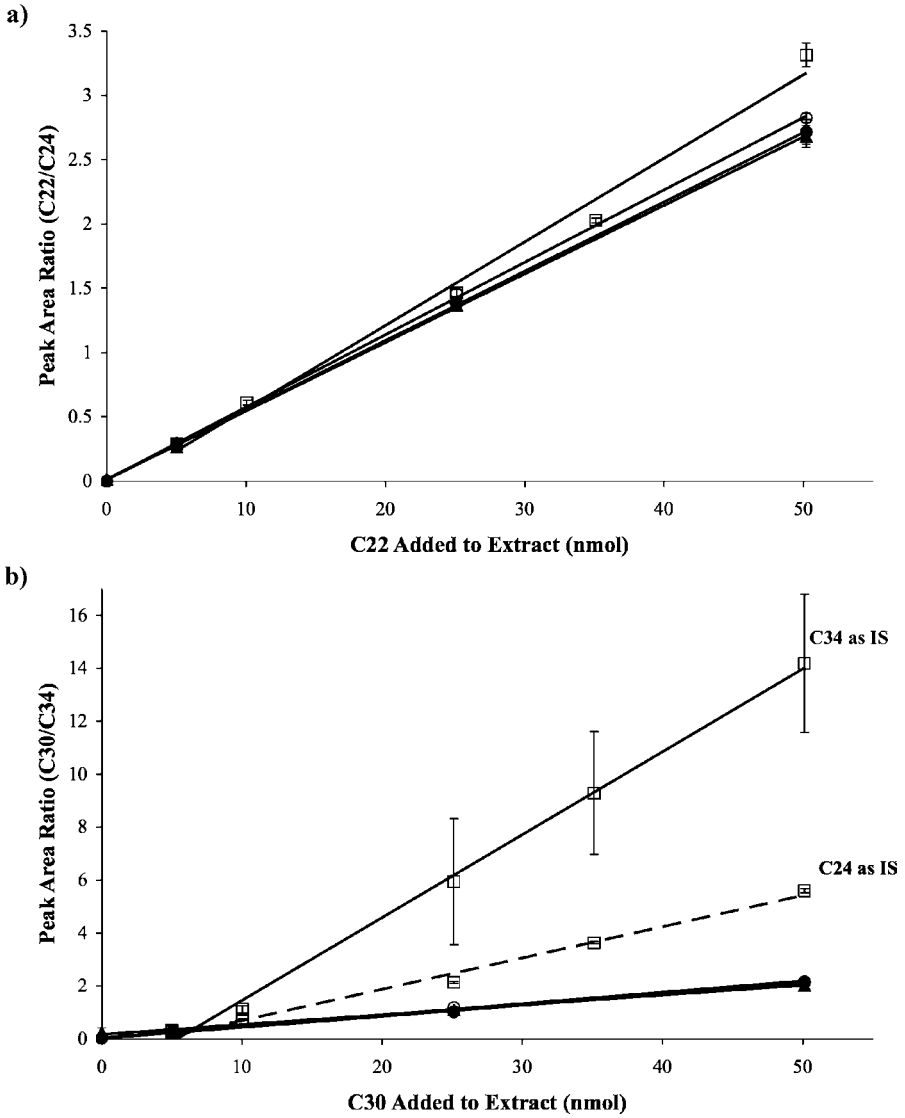

Figure 2. Standard addition calibration curves for (**a**) C22 and (**b**) C30 produced by GC/MS analysis of sample extracts (annual ryegrass (○), subterranean clover (●), and bovine feces (▲)) to which known *n*-alkane concentrations were added. Differences in linear trendline slopes for serially diluted alkane stock standard solutions (□) illustrated matrix effects manifested as ion suppression.

the internal standard is related directly to the relative detector response of C_{30} to that of C_{34} or C_{24}. For an equimolar injection, the resulting peak area for C_{30} is more similar to that of C_{24} than C_{34}. To accurately quantitate all alkanes from C_{21} to C_{36} extracted from plant and fecal tissue, it was determined that calibration curves had to be prepared using a standard addition method in which ion suppression from matrix effects would be taken into account.

Method Recovery and Extraction Efficiency. An external calibration curve prepared from working standards was used to interpolate the amount of known concentrations of alkanes that were spiked into the ASE200 extraction cells and recovered throughout the extraction and sample processing steps (**Table 2**). Complete method recovery for *n*-alkanes using the developed GC/MS method was greater than 89% for all chain lengths (21 to 36 carbon atoms). Intra-assay coefficients of variation ranged from 4.2% to 15.1%, although there was no clear correlation between carbon chain length and degree of method precision.

Accuracy. There are various general approaches by which a developed method can be evaluated for its degree of accuracy. The simplest approach is to analyze a sample of known concentration and compare its known value to the measured value. As there were no available plant or fecal samples with precisely known quantities of *n*-alkanes, the accuracy of the method had to be assessed on the basis of the quality of overall

Table 2. Complete Method Recoveries of Known Alkane Concentrations Spiked into Automated Solvent Extraction Cells

	method recovery of 30 nmol spike			
alkane	average measured concentration (nmol; $n = 9$)	exact spiked concentration (nmol)	percent recovery	intra-assay CV
$C_{21}H_{44}$	28.9 ± 2.6	31.4	92%	8.9%
$C_{22}H_{46}$	28.6 ± 1.4	31.4	91%	5.0%
$C_{23}H_{48}$	30.0 ± 1.8	31.2	96%	5.9%
$C_{25}H_{52}$	29.6 ± 1.3	31.2	95%	4.2%
$C_{26}H_{54}$	30.4 ± 1.6	31.2	97%	5.2%
$C_{27}H_{56}$	30.2 ± 1.6	31.2	97%	5.4%
$C_{28}H_{58}$	29.2 ± 2.8	31.3	94%	9.7%
$C_{29}H_{60}$	29.5 ± 2.7	31.3	94%	9.1%
$C_{30}H_{62}$	29.0 ± 2.5	31.3	93%	8.5%
$C_{31}H_{64}$	27.9 ± 2.2	31.3	89%	8.0%
$C_{32}H_{66}$	28.8 ± 2.9	31.3	92%	10.2%
$C_{33}H_{68}$	29.3 ± 4.4	31.5	93%	15.1%
$C_{35}H_{72}$	30.7 ± 2.5	31.4	98%	8.0%
$C_{36}H_{74}$	31.6 ± 4.2	31.6	100%	13.4%

Table 3. *n*-Alkane Content of Annual Ryegrass, Subterranean Clover, and Bovine Feces Using Automated Solid–Liquid Extraction and GC/MS Analysis[a]

	annual ryegrass (*Lolium rigidum*)		subterranean clover (*Trifolium subterranean*)		bovine feces	
alkane	average concentration (OM; mg/kg)	coefficient of determination (r^2)	average concentration (OM; mg/kg)	coefficient of determination (r^2)	average concentration (OM; mg/kg)	coefficient of determination (r^2)
$C_{21}H_{44}$	< LOQ		< LOQ		< LOQ	
$C_{22}H_{46}$	< LOQ		< LOQ		< LOQ	
$C_{23}H_{48}$	< LOQ		< LOQ		< LOQ	
$C_{25}H_{52}$	28.39	0.998	6.74	0.993	30.18	0.988
$C_{26}H_{54}$	8.67	0.998	< LOQ		< LOQ	
$C_{27}H_{56}$	35.52	0.996	19.08	0.993	38.19	0.988
$C_{28}H_{58}$	11.02	0.994	8.03	0.989	< LOQ	
$C_{29}H_{60}$	153.56	0.976	96.40	0.984	199.87	0.970
$C_{30}H_{62}$	11.93	0.997	5.72	0.990	< LOQ	
$C_{31}H_{64}$	229.9	0.993	28.06	0.990	309.28	0.972
$C_{32}H_{66}$	< LOQ		< LOQ		117.11	0.978
$C_{33}H_{68}$	35.38	0.992	24.23	0.981	104.43	0.980
$C_{35}H_{72}$	< LOQ		< LOQ		< LOQ	
$C_{36}H_{74}$	< LOQ		< LOQ		142.41	0.974

[a] Data from triplicate extractions per sample type were combined to generate the corresponding standard addition calibration curves. OM, organic matter; DM, dry matter; LOQ, limit of quantitation (5 nmol).

method recovery as previously discussed and comparison with literature values for the *n*-alkane content of the given samples.

The average alkane concentrations ($n = 3$) on an organic matter basis are reported in **Table 3** for annual ryegrass, subterranean clover, and bovine feces collected from an animal that had consumed a hay diet enriched with $C_{32}H_{70}$ and $C_{36}H_{74}$. The overall results for each plant sample are lower than reported literature values (*1*) determined using the original alkane analysis that involves KOH saponification, silica gel chromatographic cleanup, and analysis by GC/FID. The conditions under which the plant material is grown and at what point in the plant's lifecycle it is harvested influence the distribution of *n*-alkanes present (*12*). The analyzed samples of annual ryegrass and subterranean clover were not necessarily grown or harvested under conditions equivalent to those in the cited literature. However, the general trends for *n*-alkane content of ryegrass and clover matched well with these literature values. Specifically, more than 85% of the free alkane content was contributed by the presence of C_{27}, C_{29}, C_{31}, and C_{33}, while alkanes with even-numbered chain lengths had little appreciable concentration in the plant samples. Furthermore, the two predominant alkanes for both plant extracts were C_{29} and C_{31} with a greater concentration of C_{29} than C_{31} in subterranean clover, while the *n*-alkane distribution for ryegrass shifted to higher concentrations of C_{31} over C_{29}, in agreement with literature values. It should be noted, however, that the literature values are reported for *perennial* ryegrass, not *annual* ryegrass. This difference in plant speciation may explain any differences in the absolute *n*-alkane content. Analysis of bovine feces resulted in concentrations of C_{32} and C_{36} that were well above the detection limits of the developed method. While no actual intake estimation experiments were performed to assess the usefulness of the reported GC/MS method for estimating forage intake, the results for bovine feces are promising.

The developed GC/MS method allows *n*-alkane concentrations in plant and fecal material to be quantitated accurately and reproducibly. The incorporation of automated solid−liquid extraction at elevated temperature and pressure, combined with on-line sample filtering, reduced total analysis time for a single sample from more than 24 h to 2 h. Omitting sample cleanup using silica gel chromatography from the original method increased analysis time but resulted in matrix effects that were manifested as ion suppression. However, this ion suppression was easily overcome by using a standard addition approach to quantitation.

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