Journal of Oleo Science
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doi : 10.5650/jos.ess25041
J. Oleo Sci. **74**, (9) 825-835 (2025)

Quantification of Fatty Acids from *Arctium lappa* L. Leaves by ^{13}C qNMR and HPLC-CAD

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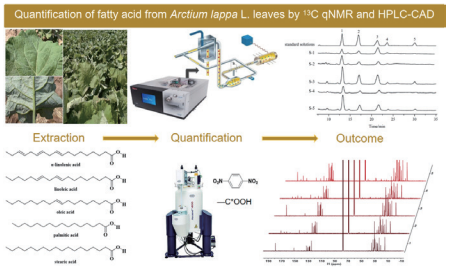
Abstract: ***Arctium lappa*** **L. (burdock) leaves is effective in the traditional treatment of stroke. Fatty acids are abundant in burdock leaves, and may play an important role in the treatment of stroke. A new procedure was presented that provides ^{13}C NMR-based quantitative measurements of total content, average chain length, average degree of unsaturation, and average polyunsaturation of fatty acids in burdock leaves from five producing areas. The content of five fatty acids in burdock leaves, including α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid, were further determined by HPLC-CAD. Among five producing areas, the results of ^{13}C qNMR showed that the content of total fatty acids in burdock leaves ranged from 8.62 to 24.51 μmol·g^{-1}, the average chain length ranged from 19.67 to 23.14, the average degree of unsaturation ranged from 1.19 to 3.91, and the average polyunsaturated moieties ranged from 0.23 to 2.37. The results of HPLC-CAD showed that the contents of α-linolenic acid, linoleic acid, palmitic acid, oleic acid and stearic acid were 0.66 - 6.60 mg·g^{-1}, 0.38-4.41 mg·g^{-1}, 0.46-3.55 mg·g^{-1}, 0.18-0.20 mg·g^{-1} and 0.10-0.32 mg·g^{-1} respectively. The developed ^{13}C qNMR method can accurately determine the total fatty acids content and provide relevant information on the fatty acids structure types in burdock leaves. This method is capable of complementing existing fatty acids quantitative methodologies, circumventing their deficiencies, and its application can be extended to other plants.**



Key words: Burdock leaves, Fatty acids, Content determination, ^{13}C qNMR, HPLC-CAD

1 Introduction

Leaves of *Arctium lappa* L., commonly known as burdock leaves, has been documented in more than twenty classic prescriptions for treating stroke in ancient Chinese medicine books[1]. "Tai Ping Sheng Hui Fang" records that "Take 500 g of burdock leaves and 50 g of clarified butter. Boil the burdock leaves in water, bringing it to a boil three to five times. Strain the leaves, prepare a soup, incorporate the clarified butter, and consume. This preparation is used to treat stroke and paralysis of the hands and feet[2]. Major constituents of burdock leaves include phenolic compounds, terpenes, and organic acids[3−5]. Traditional applications indicate that the primary therapeutic effect of burdock leaves in stroke treatment is attributed to their fat-soluble components[6]. Previous research by our group identified numerous sesquiterpenoids within burdock leaves, demonstrating significant pharmacological activity in stroke treatment[7]. Further investigations have revealed that, in addition to sesquiterpenes, the fat-soluble components also encompass a considerable amount of fatty acids. Notably, unsaturated fatty acids such as α-linolenic acid, linoleic acid, and oleic acid are crucial for human health, as they enhance blood-brain barrier permeability, regulate blood lipid levels, and help prevent cardiovascular and cerebrovascular diseases[8, 9]. We speculate that fatty acids also play a vital role in the stroke treatment of burdock

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Accepted May 30, 2025 (received for review February 18, 2025)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
https://www.jstage.jst.go.jp/browse/jos/ https://mc.manuscriptcentral.com/jjocs

leaves.

Currently, the methods reported in the literature for analyzing the composition and content of fatty acids in plant leaves predominantly utilize gas chromatography[10–12]. Relevant literature on lipid analysis of the leaves of *Arbutus unedo* and *Arbutus andrachne* using a chloroform–methanol (2:1, volume/volume) solution as the extraction solvent revealed a significant abundance of α-linolenic acid, with palmitic acid identified as the second major contributor, followed by linoleic and oleic acids[13]. The petroleum ether extract of *Haplophyllum tuberculatum* leaves was rich in γ-linolenic acid, followed by palmitic, linoleic, erucic, stearic and oleic acids[14]. The petroleum ether extracts of *Cassia tora* (leaves and stems) predominantly contained palmitic acid, linoleic acid, and linolenic acid[15]. During the experiment of gas chromatography, a series of chemical reactions are required to convert various fatty acids into their corresponding methyl esters, followed by gas chromatography analysis[16–18]. One limitation of this method is that the results do not indicate whether the plant itself contains these ester compounds or the free fatty acids have been methyl esterified. Furthermore, these pretreatment chemical reactions introduce methodological errors, imposing high technical requirements on analysts. The use of strong acids and strong bases during the pretreatment process can adversely affect the unstable olefinic bonds in unsaturated fatty acids, complicating the procedure[19]. The existing methods cannot directly measure the content of total fatty acids (tFA), so we have established a simple and accurate method to determine the content of tFA.

Nuclear Magnetic Resonance (NMR) spectrometer is an important analytical instrument in many fields[20]. It enables the acquisition of hydrogen, carbon, fluorine, phosphorus spectra, and various two-dimensional correlation spectra from samples by detecting the absorption of radio frequency radiation by atomic nuclei[21]. Its excellent resolution power to uniquely detect individual molecular species[22]. Utilizing spectral information, qualitative analysis of the structures of diverse organic compounds serves as a powerful tool for disciplines related to organic chemistry and natural medicinal chemistry[23]. Since the NMR peak integrals are directly proportional to the molecular concentration, NMR is a highly quantitative method when it comes to the determination of sample concentrations and their changes[24]. In 1D NMR, a reference compound (internal standard such as 1,4-dinitrobenzene) with known concentration can simply be added to the sample. Absolute concentrations are then obtained by relating the measured peak volumes of the compounds with unknown concentrations to the peak volume of the internal standard (IS)[25]. Since NMR spectroscopy is nondestructive, the same sample can be analyzed for an extended period of time and by different types of experiments. The preparation of NMR samples is often straightforward and may involve as little as dissolving the sample in a solution. Finally, because for the same sample essentially identical results can be obtained by different users on different spectrometers operating at the same magnetic field strength, the reproducibility of NMR data is very high[26]. The main advantages of quantitative NMR (qNMR) are that it is non-destructive, fast, reproducible and requires much less labour for sample preparation[27]. Although it has been widely used for fatty acids analysis in edible oils[28], there have been no reports on its application in the analysis of fatty acids in plant leaves.

The aim of this study is to develop a method using ^{13}C qNMR technology for analyzing the tFA content in burdock leaves. We present a new procedure that provides ^{13}C NMR-based quantitative measurements of average chain length, degree of saturation, and average polyunsaturated fatty acids content of burdock leaves from five producing areas (Xuzhou, Jiangsu; Huainan, Anhui; Jining, Shandong; Linyi, Shandong and Dingxi, Gansu). Additionally, we employed a quantitative analysis method of High Performance Liquid Chromatography with Corona Charged Aerosol Detector (HPLC-CAD) to determine the concentrations of several major fatty acids (α-linolenic acid, linoleic acid, palmitic acid, oleic acid, stearic acid) in burdock leaves for the first time.

2 Material and Methods

2.1 Chemicals and reagents

NMR spectra were measured on an Agilent 400-MR NMR spectrometer (Agilent Technologies Inc., CA, USA). An Ultimate3000 high-performance liquid chromatography system (Thermo Fisher Scientific, San Jose, CA) equipped with CAD detector and Shim-pack GIST C18 column (4.6 mm × 250 mm, 5 μm) was used to determine the content. MCI GEL (70–150 μm, Cypress (Beijing) Technology Co., Ltd.) was used for column chromatography. α-Linolenic acid (98%), Linoleic acid (98%), Palmitic acid (99%), Oleic acid (98%) and Stearic acid (98%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Ultrasonic Cleaner was purchased from Kun Shan Ultrasonic Instruments Co., Ltd (Shanghai, China). Other solvents, including methanol, dichloromethane and acetone, were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China). Further qNMR experiments were performed by using $CDCl_3$ (Rhawn, Shanghai, China) as the solvent and 1,4-dinitrobenzene (Shanghai Aladdin Biochemical Technology Co., Ltd, Shanghai, China) as the IS.

2.2 Plant materials

Burdock leaves were collected from five locations in

China: Xuzhou (Jiangsu), Huainan (Anhui), Jining (Shandong), Linyi (Shandong), and Dingxi (Gansu), coded as S-1, S-2, S-3, S-4, and S-5, respectively. Species identification was confirmed by Professor Liping Dai from Henan University of Chinese Medicine. All voucher specimens were deposited at the Classic Famous Formulas and Great Health Product R&D Center. The fresh leaves were collected, dried immediately, and stored at room temperature.

2.3 Extraction of tFA

Dichloromethane/methanol solvent was utilized to extract fatty acids from burdock leaves[29], and MCI column for impurity removal. The specific method is as follows. First, dry burdock leaves were powdered and stored in an airtight container. Subsequently, 20.0 g of the dry burdock leaves powder (in triplicate) and 200 mL of CH_2Cl_2/MeOH (5:1, v/v) solvent were added to a covered Erlenmeyer flask and subjected to sonication. The ultrasonic power was set at 80 W, with a duration of 60 minutes and a temperature maintained at 25℃. The extract was then concentrated and dried under reduced pressure. Then, it was subjected to an MCI column (2.5 × 24 cm), which was eluted sequentially using a gradient of 60% MeOH/H_2O (700 mL), followed by 95% MeOH/H_2O (1000 mL). The 95% fraction was dried under reduced pressure, dissolved in methylene chloride, and filtered through absorbent cotton. The resulting filtrate was then dried and utilized as a tFA sample for ^{13}C NMR and HPLC-CAD quantification.

2.4 NMR parameters and spectral processing

The acquisition parameters of ^{13}C qNMR was performed as reported in Reference[30] with some modifications. Briefly, s2pul Pulse Sequence, Pulse Angle (p1) = 90° flip, Relaxation Delay (d1) = 25 sec, Acquisition Time (AT) = 1.3107 sec, Number of Scans (NS) = 512, Dummy/Steady State Scans (ds) = 4, Spectral Width (SW) = 250 ppm. All NMR spectra were processed using MestReNova 12.0 software. After the Fourier transformation, phase correction and baseline correction were carefully performed. Alignment was performed using the deuterated solvents ($CDCl_3$) (delta: 77.160 ppm) signal as a reference.

2.5 Quantitative ^{13}C NMR analysis of tFA

For the preparation of the IS solution, 1,4-dinitrobenzene was dissolved in $CDCl_3$ to produce a concentration of 0.04 mol/L and utilized for all qNMR experiments. The tFA samples were dissolved in 700 μL of the IS solution and subsequently transferred to 5 mm NMR tubes. All qNMR samples were measured in accordance with the method described in Section 2.4. The resulting spectrum was analyzed using software, which marked the chemical shift values of each peak, with the integral area of the IS peak at a chemical shift of 124.9 ppm defined as 1. The integrals were obtained manually using MestReNova software and the multi-integration toolkit of AMIX-viewer. Finally, the characteristic peaks at 173.71-179.22 ppm of tFA were integrated to obtain peak area for quantification. The formula for calculating tFA acid content was as follows formula (1).

$$\text{Context (mol/g)} = \frac{I_x}{I_s} \cdot \frac{m_s}{m_x} \cdot \frac{N_s}{N_x} \cdot \frac{P_s}{M_s} \quad (1)$$

where I_s and I_x are the signal integral values of IS and tFA to be quantified, respectively. N_s and N_x represent the number of carbon nuclei of IS and tFA, respectively. And m_s and m_x refer to the mass of IS and tFA, respectively. M_s is the molar mass of IS, and P_s represents the purity of IS.

2.6 Determination of five fatty acids in burdock leaves by HPLC-CAD

2.6.1 Chromatographic methods

The chromatographic column used was a C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of methanol (A) - 0.1% formic acid water solution (B) at a flow rate of 1 mL·min^{-1} (0-20 min, 89%A; 20-20.1 min, 89%-93%A; 20.1-35 min, 93%A). The column temperature was maintained at 30℃, with an injection volume was 50 μL, the atomization temperature mode was Low, the filtration constant (filter) was 1.0 and acquisition frequency was 10 Hz.

2.6.2 Preparation of standard solutions

Accurate amounts of α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid reference substances were weighed and placed into a measuring bottle. These substances were subsequently dissolved in methanol and brought to constant volume. A single stock solution of reference substances was prepared with mass concentrations of 5.00, 60.13, 6.00, 29.70, and 5.80 g·L^{-1}. An appropriate volume of each individual reference solution was then combined in the same measuring flask, and methanol was added to adjust the volume to the mark, resulting in a reference substance stock solution at mass concentrations of 3.03, 2.28, 1.59, 0.45, and 0.43 g·L^{-1}.

2.6.3 Preparation of sample

3 g dried burdock leaves powder were taken and processed according to the method described in Section 2.3 to obtain the tFA sample. 12 mL methanol were added to dissolve the sample, which was then filtered through a 0.22 μm PTFE syringe filter for HPLC-CAD analysis.

2.6.4 Method validation

Linear relationship examination: Precisely measure the mixed reference substance stock solution under item 2.6.2, and a series of mixed reference solutions were prepared by doubling dilution method with methanol. Measure these solutions according to the chromatographic conditions outlined in item 2.2.1. The peak area was used as the ordinate and the mass concentration was used as the abscissa to draw the standard curve.

Precision test: Precisely draw an appropriate amount of the mixed reference substance stock solution under item

2.6.2, measure according to the chromatographic conditions under item 2.6.1. The samples were injected repeatedly for 6 times, and the peak area was recorded. The RSD of the peak area of α-linolenic acid, linoleic acid, palmitic acid, oleic acid and stearic acid was calculated.

Stability test: Inject the same test solution at 0, 2, 4, 6, 8, 12, 16, and 24 hours after preparation according to the chromatographic conditions under 2.6.1, record the peak area of the component to be measured, and calculate RSD of peak areas of α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid.

Sample recovery test: five fatty acids samples with known content of each index component were taken, and single reference stock solution of α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid under Section 2.6.2 were accurately added, so that the ratio of the addition amount of the five reference substances to the mass of the components to be tested in the test sample was about 1:1 (repeat 3 times). The average recovery of the above five components was calculated according to the chromatographic conditions under 2.2.1.

2.6.5 Sample content determination

S-1 to S-5 samples be prepared following the procedure outlined in section 2.6.3, and subsequently measure the samples according to the chromatographic conditions specified in section 2.6.1. The external standard method will be utilized to calculate the content of the samples.

3 Results

3.1 Fatty acids signal peak identification and quantitative peak selection in NMR spectrum

^{1}H and ^{13}C NMR spectra of tFA were interpreted based on predicted chemical shifts, literature data[19, 30, 31] and 2D NMR spectra, including HMBC, HSQC and COSY (provided in the Supporting Information). The detailed assignment of peaks is presented in Table 1. Fatty acids are carboxylic acids characterized by long hydrocarbon chains. In the structures, there are eight distinct types of hydrogen nuclei, labeled A–H (Fig. 1). Figure 2 displays the ^{13}C NMR spectra of the examined tFA and indicates the characteristic signal regions. The carbons of the methyl were observed between 14 ppm in the ^{13}C NMR spectrum, while signals from all saturated carbons located in the middle of the fatty acids chain were found between 30 and 20 ppm (labeled a in Fig. 2). Signals representing the unsaturated carbons in the fatty acids chain were found between 132 and 126 ppm (labeled b), which can be utilized to calculate the average degree of unsaturation. Signals at δ_C 180-173 ppm (labeled c) corresponds to the carboxyl carbon, which was well resolved from any other signals used for quantitation of tFA. The signals peaks around 124.90 ppm and 151.02 ppm correspond to the IS. The visual inspection of NMR spectrum where the unique signals of selected tFA, the signals from IS and the solvent were separate from each other showed specificity and selectivity of the NMR method. Based on the integration results of the signal peaks for each structural fragment in the carbon spectrum, it is also possible to calculate the average chain length and the average number of polyunsaturated moieties, as illustrated in formulas (2)–(8).

Table 1 The signals of the ^{1}H and ^{13}C NMR spectra of tFA in burdock leaves.

NO.	Description	Type of structural fragment	δ_H (ppm)	δ_C (ppm)
A, B	methyl	$-C^*H_3$	0.76-0.97	14.02-14.22
C	all carboxyl chains (saturated moieties)	$-(C^*H_2)_n-$	1.14-1.36	22.50-22.80; 28.55-30.10; 31.46-31.95
D	β-position of carboxyl	$HOOC-CH_2-C^*H_2-$	1.42-1.65	24.74-24.90
E	α-position of unsaturated and polyunsaturated moiety	$-C^*H_2-CH=CH-$	1.91-2.08	20.50-20.57; 27.16-27.29
F	α-position of carboxyl	$HOOC-C^*H_2-$	2.18-2.35	34.08-34.37
G	polyunsaturated moiety	$=HC-C^*H_2-CH=$	2.68-2.79	25.46-25.63
H	alkenyl	$-C^*H=C^*H-$	5.20-5.40	127.05-127.12; 127.68-128.30; 129.59-130.24; 131.84-131.95
I	carboxyl	$-C^*OOH$	/	173.71-174.05; 178.18-179.22

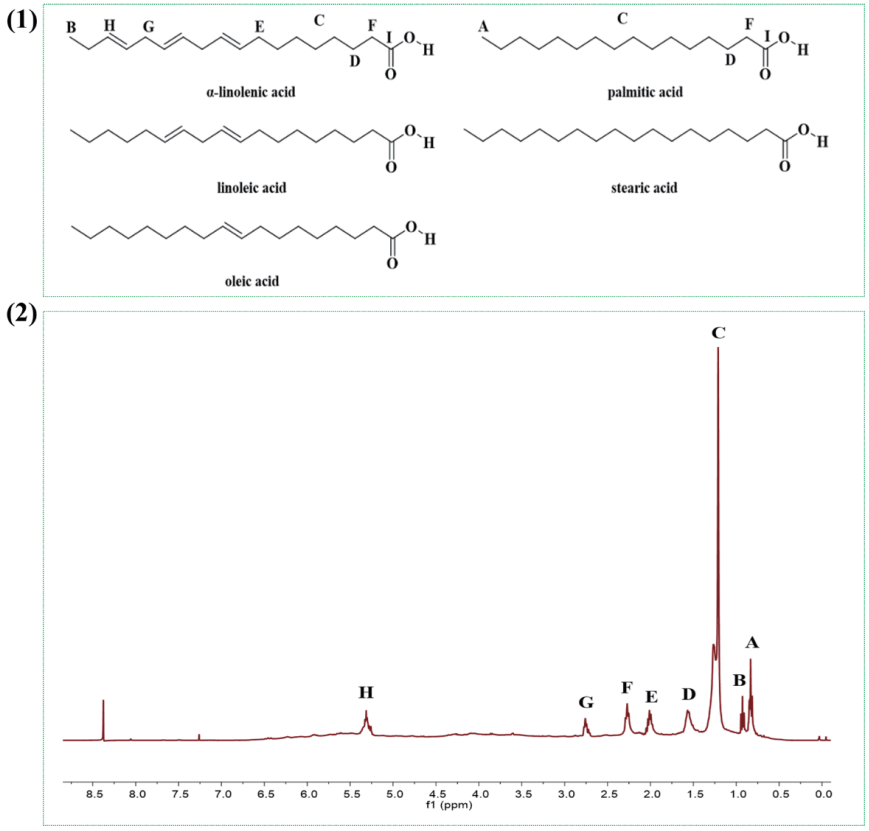

Fig. 1 (1) Structures of five predominant fatty acids in burdock leaves. Lables include methyl (A&B), saturated moiety of all carboxyl chains (C), β-position of carboxyl (D), α-position of unsaturated or polyunsaturated moiety (E), α-position of carboxyl (F), polyunsaturated moiety (G), alkenyl (H), and carboxyl (I). (2) ^{1}H NMR spectrum of tFA in burdock leaves ($CDCl_3$, 400 MHz). Peak labels follow (1).

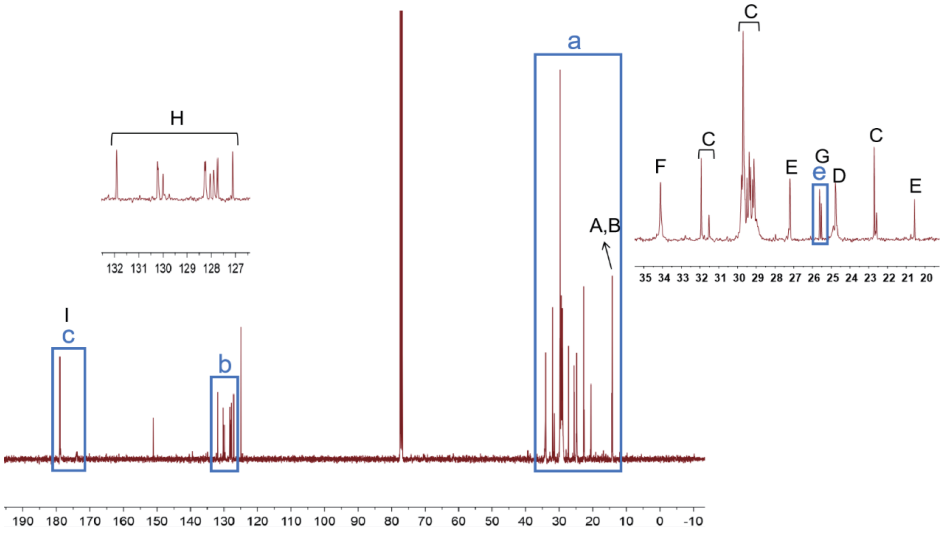

Fig. 2 ^{13}C qNMR spectrum of tFA in burdock leaves ($CDCl_3$, 100 MHz). Signal regions for all saturated carbons in the middle of the fatty acids chain (a); unsaturated carbons (b); carboxyl carbons (c); and polyunsaturated carbons (e); peak labels (A-I) follow Fig. 1.

$$a \propto \int [\text{region } 14.02 \rightarrow 34.37 \text{ ppm}] \quad (2)$$

$$b \propto \int [\text{region } 127.05 \rightarrow 131.95 \text{ ppm}] \quad (3)$$

$$c \propto \int [\text{region } 173.71 \rightarrow 179.22 \text{ ppm}] \quad (4)$$

$$e \propto \int [\text{region } 25.46 \rightarrow 25.63 \text{ ppm}] \quad (5)$$

$$\text{average chain length} = (a + b + c)/c \quad (6)$$

$$\text{average degree of unsaturation} = b/2c \quad (7)$$

$$\text{average polyunsaturated moieties} = e/c \quad (8)$$

3.2 Quantification of tFA in burdock leaves from five regions

^{13}C qNMR can be performed by integrating the -C*OOH spectral region (180-173 ppm) (labeled I) and the IS signal peak (labeled S), as illustrated in Fig. 3. The tFA content was then calculated using formula (1). The results indicated that the tFA content of burdock leaves harvested in Jining was the highest, reaching 24.09 ± 0.42 μmol·g^{-1}. Dingxi ranked second with a content of 14.84 ± 0.60 μmol·g^{-1}, followed by Linyi for 10.00 ± 0.35 μmol·g^{-1}; Xuzhou for 9.45 ± 0.06 μmol·g^{-1}; and Huainan for 9.07 ± 0.45 μmol·g^{-1}. The average chain length of fatty acids from the five locations ranged from 19.9 ± 0.23 to 22.8 ± 0.34, while the average degree of unsaturation varied from 1.20 ± 0.01 to 3.87 ± 0.04, and the average polyunsaturated moieties ranged from 0.30 ± 0.07 to 2.34 ± 0.03. The accumulation of fatty acids and the levels of unsaturated fatty acids during plant growth are influenced by factors such as pH, temperature, light intensity and nutrition[32–34]. Variations in climatic and soil conditions across different geographical regions can result in differences in the types and contents of fatty acids[35].

The tFA content in Jining burdock leaves is double that of other regions, and the average unsaturation of Jining is 3.87 ± 0.04, higher than 2.00 – 3.00 in Huainan, Linyi, Dingxi, and 1.20 ± 0.01 in Xuzhou. Its polyunsaturated fatty acids content is 2.34 ± 0.03, which is also higher than that of the other four production areas. Specifically, Huainan and Linyi have contents of 1.26 ± 0.10 and 1.43 ± 0.04, respectively, while Xuzhou and Dingxi have even lower levels at 0.68 ± 0.02 and 0.30 ± 0.07, respectively.

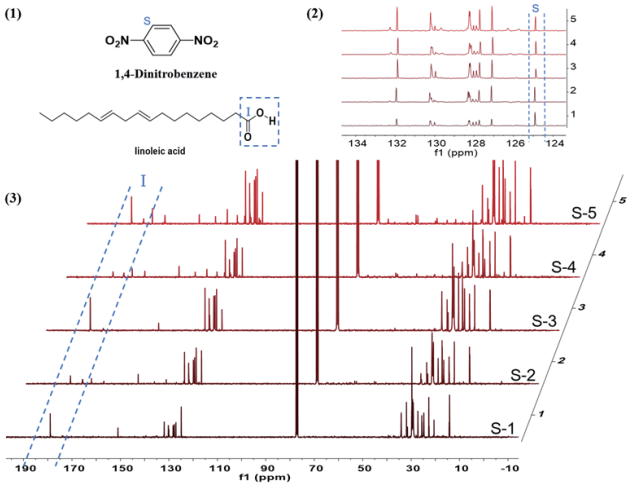

Fig. 3 (1) IS and fatty acids structure diagram. (2) Signals in ranges of 123.5–134.5 ppm in the ^{13}C qNMR spectrum of tFA sample were magnified, the blue boxes represent the quantitative signal peak of IS (S). (3) ^{13}C qNMR spectrum of tFA sample in burdock leaves from five origins in $CDCl_3$ (400 MHz), the blue boxes represent the quantitative signal peak of tFA (I).

3.3 Determination of five fatty acids in Burdock leaves by HPLC-CAD

3.3.1 Linear relationship

The test sample and reference substance were detected under 2.6.1 chromatographic conditions, and the chromatograms were recorded (Fig. 4). The standard curve was drawn with the peak area (Y) as the ordinate and the mass concentration (X) as the abscissa. The linear regression equation, linear range and correlation coefficient (r) of the 5 components were obtained (Table 3).

3.3.2 Methodological validation

The results of the methodology validation test are presented in Table 4. The RSD values for the precision and stability of the five fatty acids ranged from 0.23% to 2.90%. Additionally, the average recoveries ranged from 96.01% to 102.82%, with corresponding RSD values falling within the range of 0.45% to 2.76%. These findings indi-

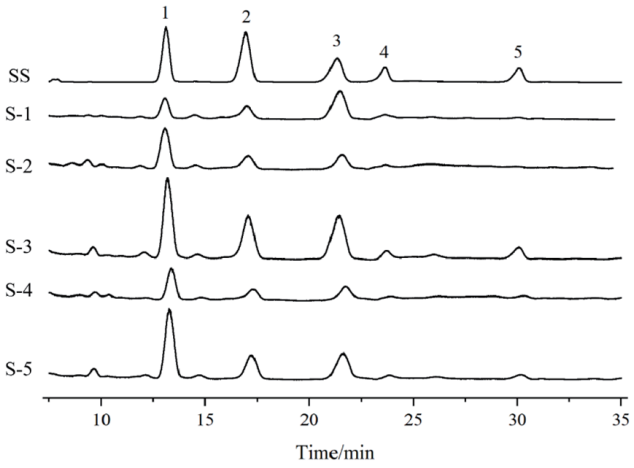

Fig. 4 HPLC-CAD spectra of standard solutions (SS) and five samples. α-linolenic acid (1), linoleic acid (2), palmitic acid (3), oleic acid (4), stearic acid (5).

cate that the developed method demonstrates accuracy.

3.3.3 Sample content determination

The quantitative results of HPLC-CAD showed that the main unsaturated fatty acids in burdock leaves are α-linolenic acid, linoleic acid, and oleic acid, while the saturated fatty acids are palmitic acid and stearic acid. The unsaturated fatty acids were dominant in burdock leaves. The fatty acids content exhibited variability depending on the geographic location. Among these five fatty acids, α-linolenic acid provides the highest concentrations in burdock leaves (1.52 ± 0.10 mg·g^{-1} for Huainan, 6.32 ± 0.28 mg·g^{-1} for Jining, 1.64 ± 0.08 mg·g^{-1} for Linyi and 4.84 ± 0.21 mg·g^{-1} for Dingxi). Accordingly, these regions demonstrated relatively high levels of average degree of unsaturation (Table 2). The palmitic acid content in burdock leaves from Xuzhou was the highest in five samples, with a concentration of 2.09 ± 0.11 mg·g^{-1}, and correspondingly, exhibited the lowest level of average degree of unsaturation among the five production areas, as detailed in Table 5. Due to the signal-to-noise ratio (S/N) of the signal peaks for oleic acid and stearic acid in the spectra from certain origins not reaching the limit of quantitation, their concen-

Table 2 ^{13}C qNMR Results.

Samples	contents of tFA (μmol·g^{-1})	average chain length	average degree of unsaturation	average polyunsaturated moieties
S-1	9.45 ± 0.06	22.8 ± 0.34	1.20 ± 0.01	0.68 ± 0.02
S-2	9.07 ± 0.45	21.6 ± 0.27	2.02 ± 0.07	1.26 ± 0.10
S-3	24.09 ± 0.42	22.8 ± 0.28	3.87 ± 0.04	2.34 ± 0.03
S-4	10.00 ± 0.35	19.9 ± 0.23	2.41 ± 0.20	1.43 ± 0.04
S-5	14.84 ± 0.60	20.9 ± 0.44	2.80 ± 0.03	0.30 ± 0.07

Table 3 Linear relationship of five fatty acids in burdock leaves.

Compounds	Regression equation	r	Linear range (mg·mL^{-1})
α-linolenic acid	*Y=103.22X+24.67*	0.9991	0.095-1.515
linoleic acid	*Y=111.58X+13.407*	0.9990	0.071-1.140
palmitic acid	*Y=158.4X+14.651*	0.9992	0.050-0.795
oleic acid	*Y=278.7X+7.046*	0.9996	0.014-0.225
stearic acid	*Y=355.15X+2.9344*	0.9998	0.007-0.108

Table 4 Methodological validation results.

Components	RSD (%)		recovery rate & RSD (%)	
	precision	stability		
α-linolenic acid	0.59	2.74	102.82	2.50
linoleic acid	0.23	2.15	96.01	0.59
palmitic acid	0.60	2.56	100.75	2.59
oleic acid	1.23	2.03	102.25	0.45
stearic acid	0.64	2.90	98.24	2.76

Table 5 Determination results of 5 fatty acids in Burdock leaves from five origins (n = 3).

Samples	Contents (mg·g^{-1})				
	α-linolenic acid	linoleic acid	palmitic acid	oleic acid	stearic acid
S-1	0.71 ± 0.05	0.81 ± 0.02	2.09 ± 0.11	/	/
S-2	1.52 ± 0.10	0.42 ± 0.04	0.52 ± 0.06	/	/
S-3	6.32 ± 0.28	4.20 ± 0.21	3.37 ± 0.18	0.19 ± 0.01	0.31 ± 0.01
S-4	1.64 ± 0.08	0.73 ± 0.06	0.53 ± 0.01	/	/
S-5	4.84 ± 0.21	1.78 ± 0.03	1.74 ± 0.13	/	0.11 ± 0.01

trations were not calculated.

The content of α-linolenic acid and linoleic acid in Jining were the highest, which were 6.32 ± 0.28 mg·g^{-1} and 4.20 ± 0.21 mg·g^{-1}. That's why Jining sample has the highest average degree of unsaturation and average polyunsaturated moieties in ^{13}C qNMR results (Table 2). In Dingxi, the contents of α-linolenic acid and linoleic acid are 4.84 ± 0.21 mg·g^{-1} and 1.78 ± 0.03 mg·g^{-1}. Therefore, the average degree of unsaturation and average polyunsaturated moieties of Dingxi are slightly lower than those of Jining, which are 2.80 and 0.30, respectively (Table 2). In conclusion, the results of HPLC-CAD are basically consistent with the ^{13}C qNMR results, suggesting that the ^{13}C qNMR method developed in this study is accurate and reliable.

4 Discussion

To the best of our knowledge, this is the first study to provide a method for rapid quantification of tFA content in burdock leaves. Though the application of ^{13}C NMR for the quantitative analysis of fatty acids in edible oils has been well-documented[36], there are currently no relevant reports on the quantitative analysis of fatty acids in plant leaves using ^{13}C NMR spectrascopy. Charles G. Fry et al.[30] established the necessary conditions of achieving accurate quantitative results from ^{13}C NMR spectral data and conducted experiments on fatty acids across various types of edible oils. QIU Qian et al.[19] utilized "anti-gated decoupling NMR technology" for quantitative testing of carbon spectra, enabling the determination of the main composition and relative content of eight types of edible oils. The complex and diverse components of plant leaves lead to interference that complicates the enrichment and quantification of fatty acids. Given that ^{13}C NMR can rapidly acquire stable signals and that sample processing is straightforward. Crucially, the characteristic peak (-C*OOH) of fatty acids shows no potential interferences due to peak overlap. Therefore, this method fulfills the quantitative requirements and is deemed viable. Through this method, we have determined the tFA content, average chain length, average degree of unsaturation, average polyunsaturated moieties in burdock leaves from five different production areas for the first time.

The fatty acids composition of the green leaves of plants generally follows a consistent pattern, with the major fatty acids being linolenic acid, linoleic acid, and palmitic acid[37]. In certain plants, significant amounts of oleic acid, stearic acid, docosanoic acid, and heptadecanoic acid are also present[38, 39]. This pattern is also observed in burdock leaves. As a universal detector, CAD offers distinct advantages in the analysis of complex mixtures. HPLC-CAD is widely employed for the qualitative and quantitative analysis of chemical components[40–42], especially carbohydrates and fatty acids[43, 44]. In this study, we selected five common fatty acids components prevalent in plant leaves and utilized HPLC-CAD to measure their concentrations in burdock leaves. This represents the first application of HPLC-CAD technology for the quantitative analysis of fatty acids from plant leaves. The measurement results indicate that burdock leaves primarily contain α-linolenic acid, linoleic acid, and palmitic acid. Combined the ^{13}C qNMR results, Jining has the highest content of unsaturated fatty acids (α-linolenic acid, linoleic acid and oleic acid), with the highest levels of average degree of unsaturation and average polyunsaturated moieties. Xuzhou has the lowest content of these three unsaturated fatty acids, with correspondingly lower levels of average degree of unsaturation and average polyunsaturated moieties. The average chain length indicates the presence of a certain amount of fatty acids components with a chain length exceeding 18 in burdock leaves. The ^{13}C qNMR results were consistent with those obtained through HPLC-CAD.

Fatty acids are essential for human cellular processes as a source of energy and for the prevention of many diseases, such as metabolic and degenerative diseases, cardiovascular and T-cell-mediated immune diseases[44, 45]. Increased dietary intake of monounsaturated or polyunsaturated fatty acids decreased the risk of experiencing a first serious cardiovascular event within a decade[46]. Plant-derived omega-3 fatty acids, specifically α-linolenic acid, has emerged as a novel cardiovascular protective agent that can enhance prognosis following experimental ischemic stroke through long-term dietary supplementation[47]. In our previous pharmacological studies, which were grounded in the traditional use of burdock leaves, demonstrated that burdock leaves extract exhibits significant activity in the treatment of stroke. In addition to the identified sesquiterpenes, we speculate that fatty acids may also play a crucial role in this process. Quantitative analysis of fatty acids in burdock leaves from five different origins revealed that those from Jining, Shandong, contained the highest concentrations of unsaturated fatty acids. This finding suggests that burdock leaves from Jining, Shandong, could be regarded as potential raw materials for the development of drugs aimed at treating stoke. This research will establish a foundation for the development of novel pharmaceuticals derived from burdock leaves.

5 Conclusion

A simpler and more accurate ^{13}C qNMR analysis method for fatty acids in burdock leaves has been established to determine the total content, average chain length, degree of unsaturation, and polyunsaturated fraction of fatty acids from five different origins. The primary advantages of this approach are: (I) There is no need for multiple reference

substances, only one internal standard substance is added; (II) The content of fatty acids can be directly determined without methyl esterification of fatty acids; (III) Non-destructive testing process samples; (IV) High repeatability, different instruments can obtain the same results with the same parameters. HPLC-CAD was further employed to quantify the contents of five fatty acids: α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid.

The primary saturated fatty acids present in burdock leaves are α-linolenic acid and linoleic acid, while palmitic acid is the main unsaturated fatty acids. Burdock leaves sourced from Jining, Shandong, exhibit the highest total fatty acids content and degree of unsaturation, as well as the highest content of the five measured fatty acids: α-linolenic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid. The results obtained from ^{13}C qNMR are largely consistent with those from HPLC-CAD. The method developed in this study is straightforward, reproducible, and accurately determines the fatty acids content of burdock leaves.

Author Contribution

Weijie Shang: Writing – original draft, Investigation, Data curation. Yanjie Huang: Writing – review & editing, Conceptualization. Yihang Zhang: Investigation. Ruojia Li: Investigation. Liping Dai: Writing – review & editing, Supervision, Funding acquisition.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (82374023), the Special Project of Traditional Chinese Medicine Research in Henan Province (2024ZYZD14), and the National Key research and Development Program of China (2017FC1701904-3).

Conflict of Interest

The authors declare there are no conflicts of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess25041

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