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Research Article

A new fluorescent derivatization reagent and its application to free fatty acid analysis in pomegranate samples using HPLC with fluorescence detection

A new fluorescent labeling reagent has been developed for the determination of fatty acids (FAs) by HPLC with fluorescence detection. The derivatization conditions including the amount of derivatization reagent, temperature, and type of catalyst were investigated, the results indicated that the reaction proceeded within 30 min at 90°C in the presence of K_2CO_3 catalyst. The maximal yield was obtained with a four- to fivefold molar reagent excess. The derivatives exhibited strong fluorescence with an excitation maximum at $\lambda_{ex} = 245$ nm and an emission maximum at $\lambda_{em} = 410$ nm. Twenty-five FA derivatives were well separated by RP-HPLC on a Hypersil BDS C_8 column in combination with gradient elution. All FAs were found to give excellent linear responses with correlation coefficients >0.9992. The method gave a low LOQ of 0.85–5.5 ng/mL (S/N of 10). The developed method was employed to analyze free FAs (FFAs) composition in pomegranate samples without any purification. FFAs in samples were doubly identified by HPLC retention time and protonated molecular ion corresponding to m/z $[M+H]^+$. This newly developed method allows a highly sensitive determination of trace FFAs from pomegranate and other foodstuffs.

Keywords: Fluorescent derivatization / Free fatty acids / Pomegranate
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1 Introduction

Fatty acids (FAs) are ubiquitous molecules typically found bound to other compounds such as glycerol, sugars, or phosphate headgroups to form lipids, which are the integral components of cell membranes. FAs can be released from lipids, typically by enzyme action, to become free fatty acids (FFAs). Several reports show that FFAs play an important role at trace levels in the regulation of a variety of physiological and biological functions [1, 2]. For example, recent studies indicate a key regulatory function of short-chain FFAs in maintaining a healthy and controlled intestinal environment [3]; FFAs have been demonstrated to act as ligands in several G protein coupled receptors, these FA receptors are proposed to play critical roles in various types of physiological homeostases [4]. There are now extensive reports concerning the antibacterial effects of various FFAs from a wide range of biological sources, including algae, animals, and plants [5–7]. Indeed, previous studies reported that FFAs are indispensable ingredients for the efficacy of medicinal plants [8, 9]. Meanwhile, they are also considered as one of the important nutritional contents in some fruits and vegetables [10]. Therefore, FFA analysis is of great importance in researching more biological activities and evaluating the nutritive value of medicinal plants and foodstuffs.

The analysis of FA mixtures was a challenging task; the most commonly used technique was to transform FAs into their less polar methyl ester derivatives (FAMEs) for analysis by GC–MS [11–14]. However, there were some obvious drawbacks of this method. For example, the derivatization reaction was tedious and time consuming [12]. Moreover, the method used acid catalysis and was not applicable to oils containing conjugated FAs due to isomerization during methyl ester preparation [13]. Besides, long-chain polyunsaturated FAs were unstable during the GC analysis because of the high temperature. Thus, the determination accuracy was greatly

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Abbreviations: **BDETS**, 2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate; **CPMS**, 1-(9H-carbazol-9-yl)propan-2-yl-methanesulfonate; **FA**, fatty acid; **FFA**, free fatty acid; **FLD**, fluorescence detection; **MPB**, 2-(7-methyl-1H-pyrazolo-[3,4-b]quinoline-1-yl)ethyl-4-methyl benzenesulfonate; **MTAD**, 4-methyl-1,2,4-triazoline-3,5-dione

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affected [14]. In contrast with the GC method, the HPLC method could overcome some problems and provide several advantages: (1) the HPLC method allows the nonvolatile, thermally labile compounds to be separated with mild operating conditions; (2) the analytes are undamaged after HPLC, which makes it possible to collect the fractions to make further investigations; and (3) an analysis method with high sensitivity and good selectivity can be developed by HPLC because of its various separation columns and different strong UV-absorbing or fluorescent tagging probes, which allow FAs to be converted to a large number of different derivatives [15–17]. As FAs have little UV and no fluorescence response, the application of direct LC methods was unfeasible for their analysis. One of the ways to deal with this problem was through precolumn or postcolumn derivatization to enhance the analytes' response. Chemical derivatization could dramatically increase the sensitivity and specificity of the GC or LC method. Many derivatization reagents have been reported, including dimethyl disulfide [18], 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) [19], 3-acyloxymethyl-1-methylpyridinium iodide [20], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone [21], 9-aminophenanthrene [22], 4-phenyl-1,2,4-triazoline-3,5-dione [23], 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) [24], 1-(9H-carbazol-9-yl)propan-2-yl-methanesulfonate (CPMS) [25], and so on. Although so many derivatization reagents have been synthesized and successfully used for FA analysis, their applications were restricted for various disadvantages such as poor stability, tedious operation, time consumption, incomplete separation, and low sensitivity.

In this study, a new fluorescence labeling reagent 2-(7-methyl-1H-pyrazolo-[3,4-b]quinoline-1-yl)ethyl-4-methyl benzenesulfonate (MPB) was synthesized, which exhibits lots of properties superior to those of the currently employed reagents in term of simple synthetic steps, excellent sensitivity, and mild sample derivatization. In addition, the derivatization conditions were further optimized by several tests as well as the separation conditions of standard derivates. Ultimately, a rapid, sensitive, and selective method with HPLC coupled to fluorescence detection (FLD) using MPB as labeling reagent was successfully developed for the simultaneous determination of FFAs in different pomegranate organs without any preliminary sample purification. This method gives detailed FFA compositions, which would be helpful in promoting work on the physiological research of FFAs in pomegranate.

2 Materials and methods

2.1 Instruments

All LC system devices were from the HP1100 series equipped with a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostatted column compartment (model G1316A), and an FLD (model G1321A). Chromatographic separation was achieved on a Hypersil BDS C_8 column (200 × 4.6 mm, 5 mm id, Dalian Elite Analytical Instruments, Dalian, China). MSD Trap-SL (ion trap) from Bruker Daltonnik (Bremen, Germany) was equipped with an ESI source (in positive ion mode). The determination of fluorescence excitation and emission spectra were performed on a 650–10S fluorescence spectrophotometer (Hitachi, Japan). The mobile phase was filtered through a 0.2 μm nylon membrane filter (Alltech, Deerfield, IL, USA).

2.2 Materials

All FAs used as standards were of chromatographic grade and purchased from Sigma Reagent (Sigma, St. Louis, MO, USA). Chloroform, DMF, methanol, hydrochloric acid, sodium hydroxide, potassium hydroxide, and anhydrous pyridine were all of analytical grade and obtained from Shanghai Chemical Reagent (Shanghai, China). HPLC-grade acetonitrile was obtained from Yucheng Chemical Reagent (Shandong province, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Pomegranate flowers were picked in May 2012. The fruit was purchased in Qufu, Shangdong province. The flowers, peel, and seeds were dried and crushed, and the juice was dried at 70°C in an oven. The derivatization reagent MPB was first synthesized in our laboratory, the synthetic steps are presented in the Supporting Information Fig. S1.

2.3 Preparation of standard solutions

The MPB solution (5.7×10^{-3} mol/L) was prepared by dissolving 21.6 mg MBP in 10 mL acetonitrile. The standard FA solutions (each 7.0×10^{-4} mol/L) were prepared by diluting the corresponding stock solution (2×10^{-2} mol/L) with acetonitrile. When not in use, all reagent solutions were stored at 4°C in a refrigerator.

2.4 Extraction procedure

0.4 g of prepared pomegranate sample (flower, peel, seed, and juice) was accurately weighed in a tube to which 10 mL of chloroform/methanol mixture (2:1, v/v) was added. The mixture was then sonicated in a bath sonicator for 60 min, centrifuged at 1000 × *g* for 5 min, and the supernatant fluid was stored at 4°C until analysis.

2.5 Derivatization procedure

The mixed standard FA solution (10 μL, each 7×10^{-4} mol/L) was added to a vial containing 30 mg of dry catalyst K_2CO_3, to which 150 μL of MPB solution (5.7×10^{-3} mol/L) and 100 μL of DMF were added and then sealed. The sealed vial was placed in a water bath at 90°C for 30 min. When the reaction was finished, 240 μL of acetonitrile was added to dilute the derivatization solution. The diluted solution (10 μL) was directly injected into the HPLC for analysis. The derivatization procedure is shown in Fig. 1. The above

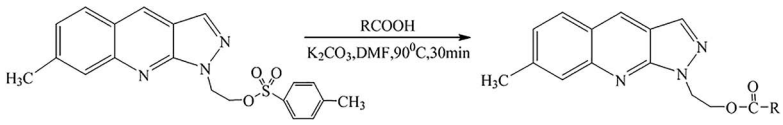

Figure 1. Derivatization of MPB with FA.

sample extract (1 mL) was precisely pipetted into a vial and dried under nitrogen. The derivatization and analytical procedures were carried out according to standards.

2.6 HPLC separation and MS condition

The mobile phase was gradient elution, which was mixed with solvent A (5% acetonitrile in water) and B (100% acetonitrile). The column temperature was kept at 30°C and the flow rate was constant at 1 mL/min. The gradient conditions of the mobile phase were as follows: 0–10 min: 40–55% B, 25 min: 70% B, 36 min: 73% B, 42 min: 83% B, 53 min: 100% B, then followed a constant elution for 7 min. The fluorescence excitation and emission wavelength was set at λ_{ex} = 245 and λ_{em} = 410 nm, respectively. The mass spectrometer 1100 series LC-MSD Trap-SL (ion trap) from Bruker Daltonik was equipped with an ESI source. Ion source conditions were as follows: ESI in positive ion mode, nebulizer pressure 241.3 kPa, dry gas temperature 350°C, dry gas flow 9.0 L/min, and capillary voltage −3500 V.

3 Results and discussion

3.1 Optimization of the derivatization conditions

The derivatization reaction was affected by various parameters, the majority of which were catalytic reagent, solvent, amount of derivatization reagent, and reaction temperature. With respect to the catalytic reagent, our previous research has proven that a basic catalyst was the best choice for the reaction between a sulfonate group and carboxylic acid group [26, 27]. In this study, several potassium salts including potassium bicarbonate, potassium carbonate, and potassium hydroxide were studied, and the results indicated the potassium carbonate was the best catalyst, which gave the highest yield. Meanwhile, further study of the amount of potassium carbonate (0–60 mg) used was also investigated, it was found that 30 mg was optimal for the derivatization. As for the reaction solvent, acetonitrile and DMF were tested, the reaction proceeded completely when both of them were added. Concerning the amount of derivatization reagent, for the representative FAs (C_{10}, $C_{18:2}$, C_{16}, and C_{21}) studied (Supporting Information Fig. S2), the derivatization yields increased with increasing the amounts of derivatization reagent, maximal yield was approached with a four- to fivefold molar reagent excess to total molar acids for all FAs, further increase of the excess reagent beyond this level had a slight effect on yields but was detrimental for the maintenance of the HPLC system. With less than a 3.0-fold molar excess of the derivatization reagent, the derivatization of FAs was insufficient and resulted in an obvious decrease of detection response. The optimum temperature was found to be 90°C by carrying out a series of experiments at different temperatures (Supporting Information Fig. S3), the reaction cannot proceed adequately at temperatures lower than 90°C, and a minor decrease of derivatization yield was observed when the temperature was higher than 90°C. Finally, a quantitative conversion was achieved owing to the complete derivatization reaction under the proposed conditions. To an unknown concentration of sample, an excess of MPB was added until a constant peak intensity was obtained to guarantee complete conversion.

3.2 Extraction of FFAs from pomegranate samples

Several analytical methods have been developed and applied for FFA extraction from foods and plants. Some single solvents such as chloroform, ethanol, and petroleum ether are commonly used for FFA extraction. However, owing to the polarity difference between the analytes and solvents, it was difficult to achieve an exhaustive FFA extraction from samples. Increasing studies reported that choosing some solvent mixtures could increase the FFA and lipid extraction yields [28, 29], a comparison showed that a chloroform/methanol mixture (2:1, v/v) was the best solvent mixture for FFA extraction [30]. On the basis of these reports, a method of ultrasonic extraction with chloroform/methanol (2:1, v/v) as solvent was carried out to extract FFAs from different organs of pomegranate. Furthermore, the sonication time and ratio of solvent to material were the main parameters that affected the extraction yields according to several reports [31], thus these two parameters were chosen for further optimization by single-factor experiments in this test. A high content of linoleic acid ($C_{18:2}$) and palmitic acid (C_{16}) existed in pomegranate flower as shown by our previous tests, these two kinds of FAs were chosen as the representatives to optimize the extraction conditions. It was found that the extraction yield increased slightly with the increase of sonication time and reached a maximum value with ultrasound for 60 min (Supporting Information Fig. S4). The increase of sonication time increased the duration of cavitation process that occurs in the extraction process, so a higher extraction yield can be obtained [31]. FFAs can be extracted sufficiently when the ratio of solvent to material was 25, less solvent would lead to an inadequate extraction, while too much has no positive effect on yield (Supporting Information Fig. S5). A high extraction yield as well as an excellent sample chromatogram with little matrix interference was achieved under the optimal conditions (ultrasonic time, 60 min; ratio of solvent to material, 25).

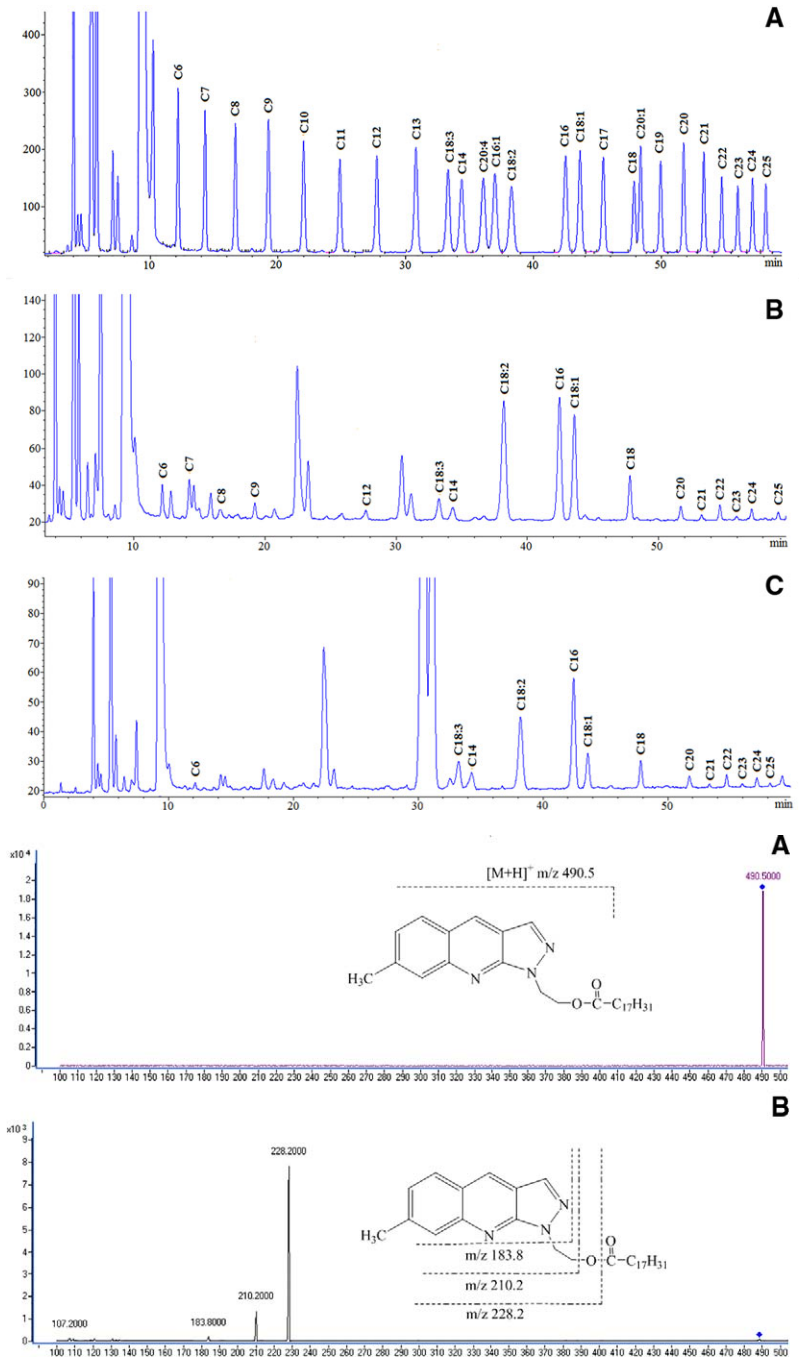

Figure 2. Typical chromatograms for the standard FA derivatives (A), FFA derivatives from pomegranate seed (B), and derivatives from pomegranate flower (C). Peak label: C_6, hexanoic acid; C_7, heptoic acid; C_8, caprylic acid; C_9, (pelargonic acid); C_{10}, decoic acid; C_{11}, undecanoic acid; C_{12}, dodecanoic acid; C_{13}, tridecanoic acid; $C_{18:3}$, 8,11,14-octadecatrienoic acid; C_{14}, myristic acid; $C_{20:4}$, 6,9,12,15-arachidonic acid; $C_{16:1}$, 9-hexadecenoic acid; $C_{18:2}$, 9,12-octadecadienoic acid; C_{16}, hexadecanoic acid; $C_{18:1}$, 12-octadecenoic acid; C_{17}, heptadecanoic acid; C_{18}, octadecanoic acid; $C_{20:1}$, 11-eicosenoic acid; C_{19}, nonadecanoic acid; C_{20}, arachidic acid; C_{21}, heneicosoic acid; C_{22}, docosanoic acid; C_{23}, tricosanoic acid; C_{24}, tetracosanoic acid; C_{25}, pentacosanoic acid.

Figure 3. MS spectra of the representative linoleic ($C_{18:2}$) derivative (A) and fragmentation pattern of the protonated molecular ion (B).

3.3 Chromatography of derivatized FAs and MS identification

The separation of 25 FA derivates by LC was studied using a Hypersil BDS C_8 column with a gradient elution. We assayed different gradients of 5% acetonitrile (in water) (A) or acetonitrile (B) at a constant flow of 1.0 mL/min, an excellent baseline separation in the shortest time was obtained using the gradients mentioned above. Finally, the optimized LC conditions allowed the concurrent separation of 25 FA derivates within 60 min (Fig. 2A). To determine the optimum conditions for MS detection, ESI was examined using the positive ion mode, which yielded the intense protonated molecular ion $[M+H]^+$ as a prominent ion. Owing to two alkalescent nitrogen atoms in the MPB molecular core, all FA derivatives produced intense molecular ion peaks at $[M+H]^+$ ions. As expected, the $C_{18:2}$ derivate produced an intense molecular ion peak at m/z 490.5 (Fig. 3). The MS data for the 25 FA derivatives are shown in Table 1. Despite the amount of other endogenous acidic compounds present in samples that were coextracted and then derivatized with the target FFAs, a clear composition of FFAs from samples could be doubly identified by

Table 1. Linearity, repeatability, LOD, LOQ, precision, MS data, and recovery for the quantification method of FAs in pomegranate samples

FA	MS data $[MH]^+$	Linearity	r	LOD (ng/mL)	LOQ (ng/mL)	Instrument precision (%, $n = 6$)		Method precision (%, $n = 3$)		Recovery (%, $n = 3$)
						Retention time	Peak area	Retention time	Peak area	
C_6	326.5	$y = 6.63x + 6.25$	0.9995	0.79	1.8	0.093	1.5	0.11	2.7	88.7 ± 2.3
C_7	340.5	$y = 7.40x - 19.69$	0.9993	0.90	2.05	0.061	0.8	0.08	1.9	92.6 ± 1.9
C_8	354.5	$y = 5.76x + 3.38$	0.9994	0.48	1.34	0.044	1.9	0.10	3.0	96.9 ± 3.0
C_9	368.5	$y = 5.24x + 9.81$	0.9996	0.96	2.77	0.032	2.3	0.12	3.8	91.7 ± 1.8
C_{10}	382.5	$y = 5.74x - 4.73$	0.9993	1.24	3.35	0.022	1.7	0.07	2.7	92.2 ± 1.3
C_{11}	396.5	$y = 4.91x + 1.77$	0.9992	0.68	1.4	0.018	2.4	0.04	3.6	90.0 ± 1.9
C_{12}	410.5	$y = 5.58\,x + 1.42$	0.9996	0.48	1.30	0.020	1.3	0.07	2.4	100.3 ± 2.8
C_{13}	424.5	$y = 5.49x + 0.53$	0.9992	1.81	3.95	0.1	2.3	0.17	3.5	91.7 ± 2.4
$C_{18:3}$	488.5	$y = 6.27x + 6.12$	0.9992	0.74	2.60	0.11	2.3	0.21	3.9	103.1 ± 3.2
C_{14}	438.5	$y = 4.41x + 13.48$	0.9993	2.5	5.39	0.029	2.8	0.13	4.1	104.4 ± 2.7
$C_{20:4}$	514.5	$y = 7.85x - 0.08$	0.9992	0.41	0.85	0.033	1.8	0.18	3.2	93.3 ± 1.6
$C_{16:1}$	464.5	$y = 5.92x + 14.14$	0.9998	0.41	0.85	0.045	2.0	0.16	5.6	90.6 ± 1.9
$C_{18:2}$	490.5	$y = 12.08x - 3.41$	0.9994	2.6	5.5	0.048	1.3	0.16	2.7	93.7 ± 2.1
C_{16}	466.5	$y = 6.74x + 14.07$	1	1.26	4.5	0.058	2.5	0.20	3.4	96.4 ± 2.8
$C_{18:1}$	492.5	$y = 10.18x + 1.17$	0.9994	0.43	1.22	0.090	2.2	0.19	3.9	92.1 ± 3.0
C_{17}	480.5	$y = 5.11x + 0.56$	0.9992	0.47	1.07	0.070	3.0	0.23	5.8	94.3 ± 1.9
C_{18}	494.5	$y = 5.\,87x - 24.31$	0.9996	0.45	1.21	0.084	2.1	0.18	3.6	89.8 ± 1.2
$C_{20:1}$	520.5	$y = 4.43x + 54.77$	0.9992	1.20	3.85	0.1	2.4	0.26	6.4	92 ± 2.0
C_{19}	508.5	$y = 4.56x + 2.99$	0.9993	0.45	1.60	0.1	1.7	0.28	5.9	90 ± 1.5
C_{20}	510.5	$y = 4.04x + 9.89$	0.9999	0.44	1.12	0.06	2.3	0.24	4.0	93 ± 2.3
C_{21}	522.5	$y = 4.04x - 4.63$	0.9999	0.43	1.11	0.07	2.1	0.21	3.8	91 ± 1.9
C_{22}	534.5	$y = 3.66x + 5.91$	0.9998	0.45	1.14	0.06	2.4	0.19	3.2	103 ± 2.1
C_{23}	546.5	$y = 3.92x - 21.10$	0.9992	0.47	1.20	0.1	3.0	0.20	3.1	90 ± 2.0
C_{24}	558.5	$y = 3.27x + 13.71$	0.9999	0.49	1.27	0.09	2.5	0.18	2.9	94 ± 3.0
C_{25}	570.5	$y = 3.18x + 6.02$	0.9998	0.41	1.32	0.08	2.2	0.20	3.0	93 ± 1.8

$y = ax + b$; y: peak area, x: molar content.
MS data, molecular weight of derivatives $[M+H]^+$.

chromatographic retention time and online MS identification for the highly specific parent mass-to-charge ratio.

3.4 Method validation

The response linearity, LOD, LOQ, precision (including instrumental precision and method precision), and recovery were validated according to the U.S. Food and Drug Administration (FDA) guidelines and several reported studies [26, 32]. As can be seen in Table 1, the calibration curves for each FA derivative were established by the derivatization of serially diluted stock standards; the linear range was from 22.6 to 8938.8 ng/mL. All FAs were found to give linear responses with correlation coefficients >0.9992. With an injection of the highly diluted standard sample, the calculated LOD (at S/N of 3:1) was from 0.41 to 2.5 ng/mL. The LOQ was obtained at S/N of 10:1 and the value was in range 0.85–5.5 ng/mL, which demonstrated the high sensitivity of this method. The precision was examined by six continuous injections of standard solution. The relative standard deviation of the retention time and peak area (RSD%; $n = 6$) were <0.018 and 3.0%, respectively. Meanwhile, the method precision was determined by adding 20 pmol standard solution to the original sample, then through the whole procedure including extraction, derivatization, separation, and quantitation to ensure the final result. The deviation of retention time and peak area (RSD%; $n = 3$) were <0.04 and 6.4%, respectively. The recoveries were calculated based on the following formula: (measured value−endogenous value)/added value × 100. All analyses were carried out in triplicate. The results indicated that the recoveries of all FAs were in the range 86.4–107.1%, which indicated that little interference existed in the whole analysis procedure.

3.5 Comparisons of the proposed method with reported methods

To demonstrate the merit of the proposed method, a comparative study with previously reported methods was carried out in terms of derivatization conditions, separation capacity, and detection limits [18–25] (Supporting Information Table S1). The dimethyl disulfide reagent was commonly used for derivatization before GC–MS analysis, but the derivatization reaction was time consuming (24 h) and the excess reagent must be removed by shaking with 5% aqueous $Na_2S_20_3$ [18]. MTAD, as a diazomethane reagent, was

Table 2. Content of FFAs in four organs of pomegranate ($n = 3$, μg/g)

FFA	Pomegranate fractions			
	Pomegranate flower	Pomegranate seed	Pomegranate peel	Pomegranate juice
C_5	nd	nd	nd	nd
C_6	1.0 ± 0.015	2.4 ± 0.04	0.47 ± 0.07	1.46 ± 0.02
C_7	nd	4.16 ± 0.03	nd	nd
C_8	1.7 ± 0.03	1.6 ± 0.03	nd	0.2 ± 0.01
C_9	nd	1.92 ± 0.04	1.89 ± 0.04	2.74 ± 0.06
C_{10}	nd	nd	nd	nd
C_{11}	nd	nd	nd	nd
C_{12}	nd	2.08 ± 0.03	nd	nd
C_{13}	nd	nd	nd	nd
$C_{18:3}$	19.72 ± 0.45	6.4 ± 0.15	10.86 ± 0.45	5.57 ± 0.23
C_{14}	11.94 ± 0.33	3.66 ± 0.1	nd	nd
$C_{20:4}$	nd	nd	nd	nd
$C_{16:1}$	nd	nd	nd	nd
$C_{18:2}$	72.95 ± 0.96	50.48 ± 0.67	12.25 ± 0.16	15.8 ± 0.42
C_{16}	69.78 ± 1.74	26.24 ± 0.66	34.6 ± 0.87	29.8 ± 0.75
$C_{18:1}$	23.17 ± 0.51	29.44 ± 0.65	32.91 ± 0.72	12.25 ± 0.3
C_{17}	nd	nd	nd	nd
C_{18}	27.6 ± 0.58	nd	5.06 ± 0.11	1.23 ± 0.03
$C_{20:1}$	nd	nd	nd	nd
C_{19}	nd	nd	nd	nd
C_{20}	7.73 ± 0.18	4.72 ± 0.11	5.03 ± 0.12	4.38 ± 0.1
C_{21}	2.43 ± 0.05	2.26 ± 0.05	2.01 ± 0.04	2.1 ± 0.04
C_{22}	13.19 ± 0.32	6.24 ± 0.15	5.34 ± 0.13	7.2 ± 0.17
C_{23}	4.91 ± 0.15	3.09 ± 0.09	2.94 ± 0.09	3.0 ± 0.09
C_{24}	11.56 ± 0.29	4.6 ± 0.12	4.5 ± 0.11	6.27 ± 0.16
C_{25}	4.59 ± 0.1	4.23 ± 0.09	3.0 ± 0.06	3.42 ± 0.07
Total content	272.27 ± 4.34	153.52 ± 1.69	120.86 ± 1.45	95.42 ± 1.33

nd, not detected.

often used to form stable conjugated FA Diels–Alder reaction products. As the FAs needed to be converted to their methyl ester derivatives and then undergo several steps to form adducts with MTAD, this procedure was very tedious [19]. The method HPLC with derivatization for FA analysis was increasingly applied instead of derivatized GC–MS owing to its several drawbacks mentioned above. 3-Bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone was one of the most used bromoalkyl reagents for the HPLC–FLD analysis of carboxylic acids. However, as the derivatization reaction was implemented in acetone or toluene in the presence of a phase-transfer agent, the excess reagent and the phase-transfer agent must be removed before injection [21]. For amine reagent 9-aminophenanthrene, the derivation conditions were mild but the solutions were toxic [22]. For the sulfonate reagents such as BDETS, CPMS, and MPB, the derivatization with carboxylic acids can be accomplished within 30 min at about 90°C in DMF with K_2CO_3 as catalyst, and the derivatization solution can be directly injected into the chromatograph for analysis [24, 25]. Compared with CPMS, a better separation can be achieved by MPB. As for BDETS, two decomposition products and lots of interferences existed in standard derivates chromatogram. Furthermore, MPB exhibited superior fluorescence properties in comparison with CPMS and BDETS. It can be seen that both maximal λ_{ex} and maximal λ_{em} of MPB derivatives exhibited about 165 nm red-shift compared with those of CPMS and BDETS derivatives, which reduced interference from the background when determining trace amounts of analytes. In addition, the HPLC sensitivity of using MPB as the derivatizing reagent was higher than those reagents. As can be seen in Supporting Information Table S1, LODs obtained with MPB were comparable or lower than those obtained with other reagents. Overall, MPB as a labeling reagent, shows good prospects in application in the analysis of carboxylic acid products in terms of stability, simplicity, and sensitivity. The simple derivatization as well as low LOD made MPB superior to the other reagents.

3.6 Sample analysis

An excess of MPB was used to guarantee the quantitative conversion of FFAs from the pomegranate extracts to their derivatives. All FFAs were reliably quantified by the developed method in this study. The total FFA contents in different organs of pomegranate are shown in Table 2. It can be seen that differences between these organs were significant: the highest value of 272.2 μg/g was in the flowers. A lower

value was observed in the juice. The values of pomegranate seeds and peel were 153.52 and 120.86 μg/g, respectively. The profile of the free unsaturated FA composition including oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$) was very similar in all organs. Among them, linoleic acid ($C_{18:2}$) was the most abundant in four organs, ranging from 12.25 to 72.95 μg/g. Meanwhile, high contents of linolenic acid ($C_{18:3}$) and oleic acid ($C_{18:1}$) were discovered in peel and flowers. The juice was also rich in oleic acid ($C_{18:1}$) with a content of 12.25 μg/g. There was an obvious difference of free saturated FA composition and molar content between various organs of pomegranate. A high content of palmitic acid (C_{16}) and stearic acid (C_{18}) was found in pomegranate flowers; the content was 69.78 and 27.6 μg/g, respectively. The peel, seed, and juice had a similar composition dominated by palmitic acid (C_{16}) with minor amounts of C_6, C_9, C_{18}, and C_{20}–C_{25}. Besides, a trace amount of C_8 also existed in peel and the seed contained a minor content of C_6–C_9 and C_{12}. High contents of C_{20}, C_{22}, and C_{24} were detected in pomegranate seed; the values were 7.73, 13.19, and 11.56 μg/g, respectively. Low contents of C_{21}, C_{23} and C_{25} were also found in seeds.

4 Concluding remarks

In conclusion, a new fluorescent labeling reagent was developed for FA determination by HPLC–FLD. This new reagent MPB has some superior properties compared to the currently used reagents. The proposed method has been successfully applied to FFA analysis in four organs of pomegranate including flower, seed, peel, and juice. The results showed that all these organs were rich in linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), oleic acid ($C_{18:1}$), and palmitic acid (C_{16}). The detailed FFA composition should be meaningful for further research and exploitation of pomegranate resources.

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