Analytical Methods

Rapid analysis of fatty acid profiles in raw nuts and seeds by microwave–ultrasonic synergistic in situ extraction–derivatisation and gas chromatography–mass spectrometry

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1. Introduction

Fatty acids (FAs) play many essential roles in biological systems including providing energy sources, serving as signaling molecules, and being the major structural components in the complex lipids of cellular membranes (Yang, Zhao, Gross, & Han, 2011). Vegetable oils generally contain a high content of unsaturated FAs, which exist as liquids at room temperature. Although unsaturated FAs are heart-healthy, they also have some undesirable properties, especially in contact with air, where unsaturated FAs can gradually become rancid or decomposed by absorbing oxygen and forming hydroperoxides (Ruiz-Jimenez, Priego-Capote, & Luque de Castro, 2004). It is well recognized that the cumbersome and time-consuming sample preparation processes associated with the analysis of large numbers of FAs samples by gas chromatography (GC) are impractical. Furthermore, it has been unambiguously confirmed that these processes are responsible for sample loss and contamination (Araujo, Nguyen, Freyland, Wang, & Kang, 2008). With this in mind, the development of a rapid, convenient and practical sample preparation method for FA profile analysis is urgently required.

Sample matrices preparation (extraction and derivatisation) represents one of the most challenging aspects of FA analysis. Efficient sample preparation can improve extraction and derivatisation and enrich the target analytes. A variety of sample preparation techniques with different solvents and methods have been developed for the extraction and derivatisation of lipids from different matrices (Milinsk, Matsushita, Visentainer, de Oliveira, & de Souza, 2008; Petrovic, Kezic, & Bolanca, 2010; Ruiz-Rodriguez, Reglero, & Ibanez, 2010). It is well known that microwave irradiation (MI) has been used as an alternative heating system in extraction oil (Ruiz-Rodriguez et al., 2010) and transesterification (Azcan & Danisman, 2008; Jeyashoke, Krisnangkura, & Chen, 1998) over the past few years. MI possesses considerable advantages compared with the conventional thermal transfer, including more effective heating, faster energy transfer, faster response to process heating control (Liu, Zhang, Mou, Hao, & Zhang, 2012; Virot, Tomao, Ginies, Visinoni, & Chemat, 2008). Ultrasonic-assisted extraction is also a fast, low-cost and efficient alternative to conventional liquid–solid extraction methods. Moreover, ultrasonic irradiation induces an effective emulsification and mass transfer which enhances the rate of ester formation under ultrasonic mixing conditions relative to conventional stirring condition (Hsiao, 2004).
Lin, Chang, & Chen, 2010; Orozco-Solano, Ruiz-jimenez, & Luque de Castro, 2010). Each method, however, has its limitations. MI, for example, tends to cause inhomogeneous heating, and ultrasonic irradiation induced lipid peroxidation (Hristov, Petrov, & Russanov, 1997). Therefore, microwave–ultrasonic synergistic extraction, which is a complementary technique involving the coupling of microwave-assisted extraction with ultrasonic-assisted extraction, has shown some more advantages (Lou et al., 2012; Cheng, Wan, Li, & Qi, 2011). There are no studies, however, that have applied this technology to accelerate derivatisation simultaneously with extraction for rapid determination of FA profiles.

Classically, fatty acid methyl esters (FAMEs) can be prepared by esterification or transesterification of vegetable oils using either acid- or base-catalysed reactions (Petrico et al., 2010). The advantages of using base-catalysed transesterification over acid-catalysed esterification have been well documented in the literature (Keera, El Sabagh, & Taman, 2011; Ruiz-Rodriguez et al., 2010), including transesterification more faster, and equipment corrosion more smaller, especially for reaction conditions much more facile (higher tolerance for water) (Velasquez-Orta, Lee, & Harvey, 2012). It has also been reported that the addition of a further solvent, such as n-hexane, is advisable for completing the methylation process in a reasonable time frame because of the poor solubility of triglycerides in methanol (Araujo et al., 2008). Several simplified methods have been reported for the analysis of FAMEs by GC (Lepage & Roy, 1986; Meier, Mjøs, Joensen, & Grahl-Nielsen, 2006; Ulberth & Henninger, 1992) and compared with the conventional multiple-step method involving a total lipid extraction followed by a laborious sequence of transmethylation, FAME extraction, concentration and final GC determination. Araujo et al. (2008) evaluated a rapid method for the quantitative analysis of FAs in various matrices with boron trihalide (BF₃ or BCl₃) in methanol followed by extraction with n-hexane. Abdulkadir et al. (2008) developed a one-step method combining extraction and esterification processes in one tube for the quantitative and qualitative analysis of FAs in marine animal samples. Their method operated at 100 °C over a period of 120 min with 14% boron trifluoride (BF₃)-methanol under continuous stirring. Li, Yue, Li, Hu, and Zhong (2009) evaluated FAs in a single zebrafish egg using ultrasonic-assisted simultaneous transmethylation and extraction in a methanolic NaOH/hexane mixture. Although these methods for FA analysis represent obvious improvements over the conventional methods, significant limitations still exist, in that the methods are invariably laborious, time-consuming, require expensive or extremely toxic agents, and suffer potential sample losses when the sample sizes are scaled down. Furthermore, there is a significant risk of oxidation and decomposition of the unsaturated FAs.

This work focused on the use of microwave–ultrasonic synergistic to promote extraction and derivatisation, effectively combining these two processes into a single procedure to establish a rapid, inexpensive, environmentally friendly, reliable, and much more operationally facile method, namely microwave–ultrasonic synergistic in situ extraction–derivatisation (MUED), for analysis of FA profiles in natural nut and seed samples. Walnut kernels (Juglans regia L.), sunflower seeds (Helianthus annuus L.), peanut kernels (Arachis hypogaea L.) and white sesame seeds (Sesamum indicum L.) were chosen as the study materials because these plant materials come large families with a worldwide distribution and belong to most important nourishing food materials and oilseed crops cultivated and utilised in most parts of the world, especially enriched in unsaturated FAs. The critical experimental parameters such as microwave power, reaction temperature, amounts of base-catalyst and derivatisation reagent (methanol) were optimised using response surface methodology (RSM).

2. Materials and methods

2.1. Chemicals and Materials

Walnut kernels (J. regia L.), sunflower seeds (H. annuus L.), peanut kernels (A. hypogaea L.), white sesame seeds (S. indicum L) were purchased from a farming by-product market in Xi’an, China. The raw material were decorticated and ground into powder with a high-speed grinder (Keweii Instrument manufacture Co., Ltd., Beijing, China) and passed through a 20-mesh sieve. The chemicals CH₃OH, n-hexane, HCl, NaCl, and KOH were purchased from the Guoyao Chemicals Co., Ltd., China. Ultra-pure water (18.2 MΩ cm) was produced by a Millipore purification system (USA) and used to prepare all aqueous solutions. All other chemicals were of analytical reagent grade.

2.2. MUED procedure

An microwave-ultrasonic synergistic XO-SM200 system with 1200 W (2450 MHz) maximum microwave power and 800 W (25 kHz) ultrasonic power (Xianou Instrument manufacture Co., Ltd., Nanjing, China) was used for the MUED procedure. The system was equipped with a reflux condenser, a magnetic stirrer bar and a contact digital thermocouple continuous feedback temperature sensor, which allowed for continuous stirring and constant temperature control in the reaction cell. The dried and ground samples (3.0 g of walnut kernel, peanut kernel, sunflower seed or white sesame, respectively) were mixed with n-hexane (45 mL), solid KOH (0.4 g) and methanol (5 mL) in a 100 mL dedicated glass reactor and irradiated at 550 W of microwave power and 360 W of ultrasonic power at a temperature of 45.0°C for a fixed reaction time of 8 min. Upon completion of the treatment, aliquots of saturated NaCl solution (10 mL) were immediately added to the reaction mixture to prevent any further derivatisation. The resulting mixture was vortex-mixed for 15 s, moderately cooled for 1 min and then centrifuged at 3500 rpm (2000g) for 3 min, with the FAMES being extracted into the upper n-hexane phase. Depending on the lipid content, the upper n-hexane phase was either concentrated under nitrogen or diluted with n-hexane, collected (1–2 mL sample) and subsequently filtered through a membrane (pore size 0.45 μm). A 1 μL aliquots of the resulting solution was injected into the GC or GC/MS systems for FAME analysis.

2.3. Conventional extraction and derivatisation procedure

Conventional Soxhlet extraction, an International Organisation for Standardisation (IOS) method was carried out as described in reference (Liu et al., 2012). The samples of 30.0 g were weighed to the nearest 10 mg and transferred to a cellulose thimble and placed in the extraction chamber. The Soxhlet apparatus, fitted with a condenser, was connected to a distillation flask containing 300 mL of solvent. Samples were thus extracted during a 4 h reflux with n-hexane. The cellulose cartridge was subsequently cooled to room temperature in a desiccator and its content then milled before being transferred again into another cellulose thimble. The procedure described above was subsequently repeated two more times for 2 h each time, giving a total extraction time of 8 h. The contents of the distillation flask were then concentrated to dryness in vacuo on a rotary evaporator at temperature 60°C and the flask was then cooled to room temperature in a desiccator and weighed to the nearest mg.

A conventional derivatisation procedure was carried out according to the standard procedure (ISO 5509, 2000). An amount of the oil, equivalent to 3.0 g of samples in the MUED method, was weighed (1.05, 2.00, 1.41 and 1.8 g of peanut kernel oil, walnut
kernel oil, sunflower seed oil, and white sesame seed oil, respectively) and dissolved in 15 mL of n-hexane, followed by the addition a certain volume (0.4, 0.7, 0.5 and 0.6 mL with the above same order, respectively) of 2 mol/L potassium hydroxide in methanol and then vortexed for 30 s. The resulting mixture was neutralized with a certain volume (0.4, 0.7, 0.5 and 0.6 mL with the above same order, respectively) of 2 mol/L hydrochloric acid and then centrifuged at 3000 rpm (1700 g) for 2 min. The upper n-hexane layer was then collected and a further charge of n-hexane (3.0 mL) was added to the tube to extract the FAMEs a second time.

The two supernatants containing the FAMEs were combined and filtered through a membrane (pore size 0.45 μm) prior to analysis. A sample of 1 μL of the filtrate was injected into the GC or GC/MS systems for FAMES analysis.

2.4. GC–MS analysis and identification of the fatty acid methyl esters

The FAMES were separated and analysed on a Shimadzu 2010 gas chromatograph (Shimadzu Corporation Analytical and Measuring Instruments Division, Kyoto, Japan) equipped with a flame ionisation detector and a 30 m × 0.25 mm i.D. 0.25-μm film RTX-5MS capillary column packed with 5% diphenyl, 95% dimethyl polysiloxane. The FAMES were identified by a QP2010 GC/MS instrument (Shimadzu, Japan). A 1-μL aliquot of FAMES was injected into the column using a 25:1 split injection port. The GC injection port, GC/MS interface, and MS source temperatures were maintained at 260, 250, and 230 °C, respectively. The oven temperature was programmed in three ramps, initially from 80 to 200 °C at 60 °C/min, then 200 °C isothermally for 4 min and finally to 230 °C at 3 °C/min after which the point the temperature was held at 230 °C for 6 min, providing a total run time of 22 min. The carrier gas was helium at a flow rate of 3.0 mL/min. The mass spectrometry detection system was operated at 70 eV full scan m/z from 40 to 500 amu. For identification of individual components, measured mass spectra were matched against the NIST27.LIB and NIST147s.LIB databases included in the GC/MS system software.

2.5. Optimisation of MUED procedure with RSM

A four-variable and three-level Box- Behnken design (BBD, a method of RSM) (Zhang et al., 2009) was applied to optimise the reaction conditions, with walnut kernels being selected as the research object. The four independent variables studied were microwave power (X_1, W), reaction temperature (X_2, °C), amount of methanol (X_3, mL), and amount of catalyst (X_4, g) and each was tested at three levels. The trial version of Design-Expert 7.1.3 (State-Ease, Inc., Minneapolis) was used for modelling and regression analysis. The total FAs peak area during GC was taken as the response (Y). In total, 27 experiments were designed and performed including 24 factorial points, and three centre points in a random order. Three replicates at the centre of the design were used to allow for estimation of a pure error sum of squares. Multiple regression analysis was performed to establish an empirical second-order polynomial model:

\[ Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \]  \hspace{1cm} (1)

In Eq. (1), \( \beta_0 \) is defined as a constant, \( \beta_i \) as the linear coefficient, \( \beta_{ii} \) as the quadratic coefficient, and \( \beta_{ij} \) as the cross-product coefficient, whereas \( X_i \) and \( X_j \) are the independent variables.

The quality of the fitted model was expressed by the coefficient of determination (\( R^2 \)) and lack of fit, and its statistical significance was checked by an F-test.

2.6. Data analysis and statistical methods

The optimisation of the ultrasonic power to the reaction mixture allowed for the achievement of the highest rates of extraction and derivatisation from the lowest possible energy input, reducing the cost of sample preparation. Hence, the effect of ultrasonic power (UP) on the MUED process was studied. The power was varied from 180 to 420 W as specified, whereas other experimental conditions were held constant for all of the reactions in this set of experiments. The results obtained are depicted in Fig. 1, the best extraction and derivatisation efficiency was obtained at 360 W of UP. When the UP was increased, the efficiency of the extraction and derivatisation initially increased with increasing power and then declined because of further enhancement of the cavitation effect. This phenomenon can be attributed to the fact that there was a lack of sufficient mixing and emulsification of the two immiscible reaction layers for the derivatisation reaction to occur at incipient levels of UP irradiation. As the UP was further increased, the efficiency of the extraction and derivatisation varied because of ultrasonic cavitations, which resulted in a larger increase in emulsification and micromixing at the phase boundary between the oil and methanol phases. Also, further increasing the power of the UP led to a reduction in the efficiency of the extraction and derivatisation processes. This can be attributed to the fact that at higher power levels, a cushioning effect is usually observed which results in decreased transfer of energy into the system and hence lower cavitation activity (Hingu, Gogate, & Rathod, 2010). Besides, side reactions, such as the saponification reaction, simultaneously occur during the transesterification and reduce the peak area of total FA.

3. Results and discussion

3.1. Selection of the ultrasonic power for MUED

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3.2. Optimisation of process parameters with BBD for MUED

Statistical experimental design and RSM analysis was used to better understand the relationships between the crucial reaction
variables and the response, and to achieve the optimal parameter conditions for the MUED procedure. RSM is a collection of mathematical and statistical techniques useful for designing experiments, building models and analysing the effects of several independent factors (Jin et al., 2012; Li & Fu, 2005). The main advantage of RSM is the requirement for the application of a reduced number of experimental trials to evaluate multiple factors and their interactions.

On the basis of “one variable at a time” experiments, a four-variable and three-level BBD was applied to optimise the reaction conditions (see Supplementary Table S1). According to these experimental results, an empirical second-order polynomial model was established Eq. (2).

\[
Y = -9.767 + 0.019X_1 + 0.176X_2 + 0.995X_3 + 3.236X_4 - 8.433 \times 10^{-5}X_1X_2 + 1.888 \times 10^{-4}X_1X_4 + 5.713 \times 10^{-1}X_2X_4 + 3.625 \times 10^{-1}X_4X_1 + 0.100X_1X_3 + 0.937X_3X_4 - 1.629 \times 10^{-3}X_1^2 - 2.078 \times 10^{-3}X_2^2 - 0.157X_4^2 - 21.062X_3^2
\]

(2)

The analysis of variance is shown in Table 1. The significance of the model was determined using an F-test. Corresponding variables are more significant if the absolute F-values are larger and the P values smaller (Chen, Wang, Zhang, & Huang, 2012). An F-value of 33.62 and P < 0.05 implied that the model was significant and there was only a 0.01% chance that a “Model F-value” this large could have been caused by noise. In contrast, the F-value for the lack of fit was only 1.26, implying that the lack of fit was not significant (P > 0.05) relative to the pure error. There was a 52.13% chance that a “Lack of Fit F-value” this large could have been caused by noise. A non-significant lack of fit was good, given that we wanted the model to fit the predicted residual sum of squares (PRESS) for the model was 1.63, indicating that the model fitted with each point in the design. Furthermore, all of the linear and quadratic parameters and four interaction parameters (X_1X_2, X_3X_4, X_1X_4, X_2X_3) were significant (P < 0.05), whereas the other interaction parameters (X_1X_3, X_2X_4) were insignificant (P > 0.05). The coefficient of determination (R^2) was 0.9751, indicating that 97.51% of the variability in the response could be explained by the model, further consolidating that the model adequately represented the real relationship between the parameters chosen. The high values of adjusted-R^2 (0.9461) implied that the model fitted the experimental data well. “Adeq Precision” measures the signal to noise ratio, with a ratio greater than 4 being desirable. Therefore, in the quadratic models of MUED, the ratios of 17.397 indicated signals adequate enough for the models to be used to navigate the design space. These results demonstrated that the optimisation using RSM yielded parameters more suitable for MUED than the classical one factor change per trial optimisation because interactions between the variables could be statistically evaluated.

It was necessary to check the interactions between the model terms because of their significance to the model equation. These interactions were clearly depicted by plotting two variables at a time on a three-dimensional surface plot. Several of these plots were created with each two targeted variables and the remainder of the variables held constant because the model contained more than two variables, as shown in Fig. 2 (A–F).

Fig. 2(A) illustrates the interaction between microwave power and the reaction temperature. At a fixed amount of methanol (4 mL) and amount of catalyst (0.3 g), the response first increased with increasing temperature and then declined with further heating. An increase in the reaction temperature resulted in enhanced extraction of the lipids and the solubility of the methanol/lipid mixture in the n-hexane phase, thereby increasing the efficiency of extraction and derivatisation. Further increases in the reaction temperatures and microwave power, however, resulted in lower extraction and derivatisation efficiency. This may have been due to the fact that the oil/lipid and the methyl esters tended to oxidise or become thermally unstable alky esters owing to the high content of unsaturated FAs at high microwave power. The microwave power and reaction temperature clearly exhibited a quadratic and interaction effect on the response, with the maximum response being obtained at 500–600 W and 45–52.5 °C.

Fig. 2(B) portrays the effect of microwave power and amount of methanol at a reaction temperature of 45 °C and catalyst amount of 0.3 g. The response increased initially, and then decreased at a higher microwave power and amount of methanol, most likely because of initially enhancing mass transfer and later accelerating solvent volatilisation or decomposition/saponification and oxidation of the oil or FAMES with increasing microwave power. An obvious quadratic term effect of microwave power and amount of methanol was observed under the test condition, and the maximum response was obtained at 500–600 W and 4–5 mL methanol.

Fig. 2(C) depicts the effect of microwave power and amount of catalyst. At the given reaction temperature (45 °C) and amount of methanol (4 mL), the response increased initially, and then decreased at a higher microwave power and catalyst amount. The maximum response was obtained at 500–600 W and 0.3–0.4 g catalyst. Microwave power and the amount of catalyst also demonstrated a quadratic effect on the response, likely because of the significant interaction (P < 0.05) between microwave power and the amount of catalyst.

Fig. 2(D) describes the effect of reaction temperature and amount of methanol. The maximum response being obtained at 45–52.5 °C and 4–5 mL methanol. It was observed that the efficiency of the extraction and derivatisation notably declined at high temperatures. This phenomenon was chiefly attributed to the tendency of the unsaturated FAs to decompose via the isomerization of the double bond functional group from the cis-type of C=C double bond to the corresponding trans-type and oxidation of the double bond. Volatilization of the methanol could also have had a significant impact.

Fig. 2(E–F) delineates the effect of reaction temperature and amount of catalyst and the effect of the amounts of methanol and catalyst, respectively. The data were orderly and showed a
The studentized residuals were calculated to check the adequacy of the model. These residuals represented the difference between the actual response value and the best fitted value under the hypothesized model (Chou, Norli, & Anees, 2010). In this study, the residual values were small for MUED, which specified that the predictions of the model were accurate. The normal probability plot of the standardised residuals (see Supplementary Fig. S1 (a)) showed that the response fitted well with the experimental data and that there was no abnormality in this study. The residuals versus the predicted values of MUED (see Supplementary Fig. S1 (b)) revealed that the residuals were randomly scattered at around ±1.5, which was an indication of the fit of the experimental data with the predicted data (obtained from the model). The predicted versus actual plots for MUED indicated that the observed points on the plots revealed that the experimental values were distributed relatively near to the straight line and showed satisfactory correlation between these values (see Supplementary Fig. S1 (c)). Cook’s distance plots indicated that there was no point that was potentially powerful because of the location in the factor for the MUED (see Supplementary Fig. S1 (d)).

To obtain the optimal MUED conditions, a further calculation was conducted according to the model equation (2). The optimal conditions obtained included a microwave power of 547.70 W, reaction temperature of 44.62 °C, methanol volume of 5.11 mL and catalyst amount of 0.37 g. For the sake of convenience, the actual reaction conditions chosen were a microwave power of 300.00–400.00 W, reaction temperature of 30.00–37.50 °C, methanol volume of 2.00–3.00 mL and catalyst amount of 0.10–0.20 g. The studentized residuals were calculated to check the adequacy of the model. These residuals represented the difference between the actual response value and the best fitted value under the hypothesized model (Chou, Norli, & Anees, 2010). In this study, the residual values were small for MUED, which specified that the predictions of the model were accurate. The normal probability plot of the standardised residuals (see Supplementary Fig. S1 (a)) showed that the response fitted well with the experimental data and that there was no abnormality in this study. The residuals versus the predicted values of MUED (see Supplementary Fig. S1 (b)) revealed that the residuals were randomly scattered at around ±1.5, which was an indication of the fit of the experimental data with the predicted data (obtained from the model). The predicted versus actual plots for MUED indicated that the observed points on the plots revealed that the experimental values were distributed relatively near to the straight line and showed satisfactory correlation between these values (see Supplementary Fig. S1 (c)). Cook’s distance plots indicated that there was no point that was potentially powerful because of the location in the factor for the MUED (see Supplementary Fig. S1 (d)).

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Fig. 2. Response surface plots and analysis of variance for the optimisation of the MUED process. (A) Interaction between microwave power ($X_1$, W) and reaction temperature ($X_2$, °C); (B) Effect of microwave power ($X_1$, W) and amount of methanol ($X_3$, mL); (C) Interaction of microwave power ($X_1$, W) and amount of catalyst ($X_4$, g); (D) Effect of reaction temperature ($X_2$, °C) and amount of methanol ($X_3$, mL); (E) Interaction of reaction temperature ($X_2$, °C) and amount of catalyst ($X_4$, g); (F) Interaction of amount of methanol ($X_3$, mL) and amount of catalyst ($X_4$, g).
550.0 W, reaction temperature of 45.0 °C, methanol volume of 5.0 mL and catalyst amount of 0.4 g. The chosen experimental conditions were validated with the model equation. A total peak area of \((2.298 \pm 0.013) \times 10^6\) \((n = 3)\) was obtained from the experiments, which was in close agreement with the value of \(2.371 \times 10^6\) predicted by the model equation.

### 3.3. Selection of optimal reaction time for MUED

The amount of FAMEs from in situ extraction-derivatisation for analytes depended on the mass transfer and equilibrium distribution processes of analytes from the plant cells and tissues into the solvent and the interaction of analytes with the derivatisation reagent. It is clear from Fig. 3, that the total chromatographic peak areas of FA (saturated and unsaturated) and peak areas of individual FAs increased with extending MUED time across a reasonable range, and that longer time resulted in an obvious decrease. The optimal reaction time of MUED of 8 min appeared to be adequate for the complete extraction and derivatisation reaction under microwave and ultrasonic irradiation, evidently shorter than the conventional method, which required more than 8 h. This disparity can be explained as followed. First and foremost, thermal and emulsification effects caused by the microwave and ultrasonic irradiation led to an increase the extractive properties of \(n\)-hexane towards the extraction of the oils from the sample matrix in suspension (diffusive extraction). Furthermore, extended microwave and ultrasonic effects led to penetration through the cell walls, forcing the oils out into the solvent mixture (disruptive extraction). Sufficient thermal transfer and emulsification can be explained as followed. First and foremost, thermal and emulsification effects caused by the microwave and ultrasonic irradiation led to an increase the extractive properties of \(n\)-hexane towards the extraction of the oils from the sample matrix in suspension (diffusive extraction). Furthermore, extended microwave and ultrasonic effects led to penetration through the cell walls, forcing the oils out into the solvent mixture (disruptive extraction). Sufficient thermal transfer and emulsification can be explained as followed. First and foremost, thermal and emulsification effects caused by the microwave and ultrasonic irradiation led to an increase the extractive properties of \(n\)-hexane towards the extraction of the oils from the sample matrix in suspension (diffusive extraction). Furthermore, extended microwave and ultrasonic effects led to penetration through the cell walls, forcing the oils out into the solvent mixture (disruptive extraction).

### 3.4. Application of the method to the analysis of fatty acid profiles in four nuts and seeds

The typical GC/MS total ion chromatograms from the MUED were compared with conventional extraction-derivatisation (see Supplementary Fig. S2 (a–d)). The total chromatographic peak areas of the FAs from equivalent amounts of peanut kernels, walnut kernels, sunflower seeds, and white sesame seed samples were \((5.988 \pm 0.043) \times 10^7\), \((8.885 \pm 0.021) \times 10^7\), \((5.541 \pm 0.021) \times 10^7\) and \((7.284 \pm 0.041) \times 10^7\) for the MUED method and \((4.510 \pm 0.031) \times 10^7\), \((6.693 \pm 0.040) \times 10^7\), \((4.406 \pm 0.069) \times 10^7\) and \((6.433 \pm 0.087) \times 10^7\) for the conventional method, respectively. The results showed that MUED exhibited a higher efficiency of extraction and derivatisation than the conventional method \((P < 0.05)\).

The individual FA percentage compositions (\%) of all samples obtained by the MUED and conventional methods are presented in Table 2 with statistical comparisons. In total, nine primary FAs were identified in the peanut kernels using the MUED method, eight primary FAs in the walnut kernels, seven major FAs in the sunflower seeds and eight staple FAs in white sesame seed samples. From the conventional method, seven FAs were identified in the peanut kernels, six in walnut kernels and sunflower seeds and eight in the white sesame seed samples. The predominant FAs were \(c16:1\), \(16:0\), \(18:0\), \(c18:1\), \(c18:2\), \(c18:3\), \(20:1\), \(20:0\) and \(22:0\).

Table 2 shows that the distribution of individual FA (as a percentage) obtained by the MUED and conventional methods. The composition of individual FAs in each sample was calculated by comparing the peak area of each FA with the total peak area of all FAs in the sample. The research results showed that the total FA (TFA) percentage compositions (%) were higher in the MUED method than the conventional method. Moreover, the mono-unsaturated FA (MUFA), polyunsaturated FA (PUFA) and total unsaturated FA (TUF) contents obtained from the MUED method were clearly higher than those obtained from the conventional method \((P < 0.01)\). Interestingly, the content of saturated FAs (SFA) obtained by MUED method was markedly lower than that obtained by conventional method in all samples \((P < 0.01)\). The results could be explained by the partial decomposition or oxidation of the unsaturated FAs in the conventional method, as described by previous report (Liu et al. 2012). Significant differences were observed in the classes of FAs found for SFA, MUFA, PUFA, TUF and TFA in all samples. Significantly higher FAs content were also found for 16:1, c18:1, c18:2, and \(c18:3\) using the MUED method in all samples, with the exception of 18:0 and 20:0 in the peanut kernel, 16:0 and 18:0 in the white sesame seed, and 18:0 in the walnut kernel and sunflower seed samples. Thus, it can be further concluded that the MUED method not only provided higher derivatisation efficiency, but also had lower rates of oxidation and decomposition of the unsaturated FAs than the conventional method. Additionally, trace amounts of the long chain FAs could be accurately detected by MUED method whilst the only parts of long chain FAs were determined using conventional method.

The precision of the MUED method was also evaluated with six replicate experiments involving each of the samples. The results showed that the MUED analysis of the FAs generated reproducible profiles (Table 2), With the exception of the unsaturated and saturated derivatives (c16:1, c18:3 and 20:0), which presented relative standard deviations (RSD) between 7% and 15%, the RSD values in peanut kernel samples were below 4%. In contrast, the conventional method provided relatively poor precision, with the RSD \((n = 4)\) values for 16:1, 20:1 and 20:0 being 21.08, 13.26 and 10.05%, respectively. For walnut kernel samples, the RSD values were below 2%, except for saturated and unsaturated derivatives (20:0, 20:1 and 20:4), which presented RSD values between 16 and 25%. In contrast, the conventional method gave relatively

![Fig. 3. Effect of reaction time on the MUED method. The reaction conditions: walnut kernel sample (3.0 g), \(n\)-hexane (45 mL), methanol (5 mL), and KOH (0.4 g) with a microwave power of 550 W and ultrasonic power of 360 W at a temperature of 45 °C (Results are expressed as mean ± SD, \(n = 3\)).](image-url)
low precision and few target detection results, with the RSD ($n = 4$) value for 20:1 being 50.0%. With regard to sunflower seed samples, the RSD values were below 2%, except for 20:1, 20:0 and 22:0 with RSD values of 10.54%, 9.10% and 8.96%, respectively. In contrast, the conventional method provided relatively undesirable levels of precision, with the RSD ($n = 4$) values for 20:1 and 20:0 being 25% and 25.14%, respectively. Similarly, for the white sesame seed samples, the RSD values were below 3%, except for 16:1, 20:1 and
20:0 with an RSD values between 7% and 18%. In contrast, the conventional method gave relatively poor precision, with the RSD values for unsaturated derivatives (c16:1, c18:3, 20:1 and 22:0) being between 12% and 57%. These results can be attributed to the degradation of the unsaturated FAs via decomposition and oxidation pathways in the conventional method. It was also observed that the absence of a thorough contact between reagent and sample resulted in a low yield and poor precision, as described by Reisa, dos Reisa, Leatha, and Stelwagen (2011).

3.5. Repeatability and stability for the four samples

Within-laboratory repeatability was evaluated with all samples subjected to the proposed method. Two analyses of these samples per day were carried out for 3 days (n = 5). The variance caused by inter-day repeatability (Orozco-Solano et al., 2010) was determined by Eq. (3) as follows:

$$S_r^2 = MS_b - MS_w \over n_t$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and n_t is the number of replicates per day. The within-laboratory reproducibility, S_WR², was calculated by Eq. (4) as follows:

$$S_{WR}^2 = S_r^2 + S_b^2$$

where S_r² is the residual mean squares within-days and S_b² is the variance caused by the inter-day effect.

For analysts 1 and 2, the results showed that the between-day repeatability and within-laboratory reproducibility, which were both expressed as RSD, ranged from 3.4% to 5.3% and 6.2% to 10.7% (Table 3). Comparisons of the determination results for four samples with two analysts indicated good repeatability and within-laboratory reproducibility, which were both expressed as RSD, ranging from 3.4% to 5.3% and 6.2% to 10.7%. These results can be attributed to the degradation of the unsaturated FAs via decomposition and oxidation pathways in the conventional method. It was also observed that the absence of a thorough contact between reagent and sample resulted in a low yield and poor precision, as described by Reisa, dos Reisa, Leatha, and Stelwagen (2011).

### References


