Identification and characterization of matrix components in spinach during QuEChERS sample preparation for pesticide residue analysis by LC–ESI–MS/MS, GC–MS and UPLC–DAD

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Abstract In this article matrix components in spinach were investigated in detail. The samples were prepared using two QuEChERS (quick, easy, cheap, effective, rugged and safe) methods, AOAC and CEN. Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), gas chromatography–mass spectrometry (GC–MS) and ultra performance liquid chromatography–diode array detector (UPLC–DAD), were applied for component identification. The strategies of identification by LC–ESI–MS/MS include accurate mass spectra of the parent ion, comparison with previous literature data and investigation of the mass spectral decomposition pattern. Overall, fourteen components were identified by LC–ESI–MS/MS in each methods of AOAC and CEN, which were phytosteroids, flavonoids, fatty acids and fatty acid amides. Fifty components using AOAC method and fifty-seven components using CEN method were identified in GC–MS by comparing mass data with the National Institute of Standards and Technology (NIST, U.S.) database. The results indicate that the major components of the matrix are terpenoids, fatty acids and fatty acid esters. Moreover, three pigments (neoxanthin, violaxanthin and lutein) in the AOAC method and eight pigments (neoxanthin, violaxanthin, zeaxanthin, lutein, chlorophyll *a*, chlorophyll *b*, pheophytin *a* and beta-

carotene) in the CEN method that gave a characteristics peak at 440 nm were identified by the UPLC–DAD. According to the sample preparation condition using different amounts of graphitized carbon black (GCB) in this study, the AOAC method had higher matrix component removal efficiency than the CEN method. A better understanding of matrix components would increase the current knowledge for improvement of existing QuEChERS methodology, as well as contribute to new method developments.

Keywords Matrix components · LC–ESI–MS/MS · GC–MS · UPLC–DAD · Flavonoids · Pigments

Introduction

Due to the ever growing agricultural practices, the amount of pesticide consumption has increased worldwide (Erich-Christian and Heinz-Wilhelm 2004). Pesticides are used for the control of pests, weeds and insects, with the intension of improving crop productivity. Aside from the positive attributes, plant protection products residues remain in food stuffs, which pose health problems to consumers. Therefore, the concentration of pesticide must be monitored properly to ensure a safe food supply. High performance liquid chromatography with diode array detector (HPLC–DAD) and gas chromatography coupled with electron capture detector (GC–ECD), flame photometric detector (GC–FPD) and nitrogen phosphorus detector (GC–NPD) are sometimes used to monitor pesticide residues (Antonia et al. 2009). However, residue level determination by conventional detectors often suffers from lack of selectivity and sensitivity (Xiu et al. 2015). In recent years, mass spectrometry has been attracting growing interest for

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quantitative and qualitative analysis of residue chemicals. LC–MS/MS and GC–MS/MS enable operation either in selected ion monitoring (SIM) or multiple reaction monitoring (MRM) modes, which facilitates higher selectivity and sensitivity, thus eventually creating a wider analytical scope of target compound analyses (Anneli et al. 2008).

Although, LC–MS/MS and GC–MS/MS are being embraced as the most sophisticated and powerful tools in analytical techniques, both instruments respond adversely during target compound analysis in the MS detector due to the matrix effect, one of the drawbacks leading to the determination of an inaccurate analyte concentration (Steven et al. 2010). The matrix effect refers to the change of analyte response due to the presence of other component in the sample (Helen et al. 2012).

Since the development of the QuEChERS (quick, easy, cheap, effective, rugged and safe) method by Michelangelo et al. (2003), it has been readily accepted worldwide for pesticide analysis (Michelangelo et al. 2003; Kuniyo et al. 2015). This method has several advantages over traditional methods and has been adopted by chemical analysts for a variety of sample preparations, including for fruits and vegetables (Steven et al. 2005). The Original QuEChERS method have been modified to improve analyte and samples scope. Recently, two versions of the QuEChERS method have been predominantly used, which are the AOAC and CEN methods.

In the present study, the spinach sample was prepared as per the QuEChERS AOAC Official Method 2007. 01 (Steven 2007) and CEN standard Method EN 15662 (European committee for standardization 2008). In the AOAC method, 1% acetic acid in acetonitrile is used for extraction, and in the CEN method, only acetonitrile is used. During cleanup, 150 mg magnesium sulfate MgSO_4 , 50 mg primary secondary amine (PSA) and 50 mg graphitized carbon black (GCB) is used per ml of extract in the AOAC method. For highly pigmented samples recommended that the amount of GCB be 50 mg for the cleanup of 1 ml of extract for the AOAC when there are no planar pesticides among the analytes (Steven 2007; Tomasz and Tomasz 2015). The AOAC method for spinach sample preparation was followed in literature elsewhere (Kuniyo et al. 2012a, b; Tomasz and Tomasz 2015; Zulaihat et al. 2015). Previously, a 150 mg MgSO_4 , 25 mg PSA and 7.5 mg GCB mixture was used for each ml cleanup in the CEN method (Kuniyo et al. 2012a, b; Steven et al. 2010; Tomasz and Tomasz 2015) for different commodities, including spinach, with several reports published (Kuniyo et al. 2012a, b; Mei et al. 2014; Tomasz and Tomasz 2015).

Though numerous studies have been conducted on plant component identification, only a few studies focus on matrix components during pesticide residue analysis (Kuniyo et al. 2012a, b, 2015), and none detail the

identification of matrix components in any sample. This study adopted matrix components identification by combined application of LC–ESI–MS/MS, GC–MS and UPLC–DAD for spinach as a representative sample commodity. If the remaining matrix components are identified, we could efficiently remove them, which is important to eliminate matrix components and for optimization of existing QuEChERS methods. Identification of the remaining components is important to differentiate characteristics between matrix components with pesticide residues, as well as to provide a better cleanup.

Materials and methods

Plant material

Fresh spinach was collected from a local super market in Jeonju, South Korea. The roots were cut off, and the rest chopped and comminuted in frozen conditions with dry ice using a mixer machine (Artlon gold DA-338G, South Korea). The sample was stored in the refrigerator at -20°C until use.

Chemicals

HPLC grade acetonitrile was obtained from Sigma Aldrich (St. Louis, MO, USA), and high purity formic acid was obtained from Merck (Darmstadt, Germany). Ultrapure water was provided by a Milli-Q system from a Millipore Corp. (Bedford, MA, USA) water purification system. The following pigment standards were purchased such as α -carotene, β -carotene, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, pheophytin *a*, α -cryptoxanthin, β -cryptoxanthin, chlorophyll *a* and chlorophyll *b* from DHI Lab products (Hoersholm, Denmark). α -tocopherol and γ -tocopherol were obtained from Sigma Aldrich (St. Louis, MO, USA).

The Bond Elut Extraction packets were purchased from Agilent technologies (Lake Forest, CA, USA), which consist of a mixture of 6 g anhydrous MgSO_4 + 1.5 g anhydrous sodium acetate (NaOAc), and 4 g MgSO_4 + 1 g sodium chloride (NaCl) + 1 g trisodium citrate dihydrate ($\text{Na}_3\text{Cit} \cdot 2\text{H}_2\text{O}$) + 0.5 g disodium hydrogencitrate sesquihydrate ($\text{Na}_2\text{HCit} \cdot 1.5\text{H}_2\text{O}$) for the AOAC and CEN sample preparations, respectively. Each 15 ml Bond Elut polypropylene centrifuge tube had a d-SPE cleanup AOAC kit with 1200 mg MgSO_4 + 400 mg PSA and each CEN d-SPE kit had 900 mg MgSO_4 + 150 mg PSA, obtained from Agilent technologies (Lake Forest, CA, USA). GCB 120/400 mesh was bought from Supelco (Bellfonte, PA, USA), which was added separately to the d-SPE cleanup tubes, in which each ml of extract contained 50 and 7.5 mg GCB, respectively, for AOAC and CEN.

Sample processing and preparation

The AOAC and CEN procedure for the sample preparations are as follows: (1) weigh 15 g (AOAC), or 10 g (CEN) commuted sample in two 50 ml polypropylene tubes; (2) add 15 ml 1% glacial acetic acid in acetonitrile (MeCN) for AOAC, and add 10 ml MeCN for CEN; (3) place the mixture in a rotary shaker and shake vigorously for 15 min at the rate of 250 rpm; (4) add the extraction mixture that contains, 6 g anh. MgSO_4 + 1.5 g NaOAc (AOAC method) and 4 g anh. MgSO_4 + 1 g NaCl + 1 g $\text{Na}_3\text{Cit}\cdot 2\text{H}_2\text{O}$ + 0.5 g $\text{Na}_2\text{HCit}\cdot 1.5\text{H}_2\text{O}$ (CEN method); (5) shake the tubes immediately and vigorously by hand for 1 min; (6) centrifuge the tubes at 3000 rpm for 3 min; (7) transfer 8 ml of extract into the d-SPE tubes containing 1200 mg MgSO_4 + 400 mg PSA + 400 mg GCB (AOAC method) or transfer 6 ml extract to 900 mg MgSO_4 + 150 mg PSA + 45 mg GCB (CEN method); (8) vortex the d-SPE tubes for 30 s, and centrifuge at 3000 rpm for 3 min; and (9) collect the extract in separate clean empty tubes for component analysis.

Analytical condition for LC-ESI-MS and LC-ESI-MS/MS

For the LC-ESI-MS/MS instrument, an Applied Biosystems 3200 QTRAP (Toronto, ON, Canada) MS/MS system was adjusted to an Agilent 1100 LC, equipped with an autosampler, a vacuum degasser, a binary pump and a column temperature controller. The analytical column, Waters Atlantis T3 C18 (3.0 $\times$ 150 mm, 3 μm particle size; Milford, MA, USA) was connected with a C18 security guard cartridge for chromatographic separations. The column oven temperature was 40 $^\circ\text{C}$, the injection volume was 5 μL , and the mobile phase flow rate was 0.2 ml/min. In positive ion mode, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. In negative ion mode, mobile phase A was 0.01% formic acid in water, and mobile phase B was 0.01% formic acid in acetonitrile. In each mode, positive or negative, gradient separation was achieved by 22% B at 0 min, which was ramped to 85% over the course of 15 min, followed by a linear gradient to 99% up to 35 min, which was maintained until 50 min, then reached 22% up to 53 min to return to the initial state and remained unchanged until 60 min.

Nitrogen was generated as the nebulizer and collision gas. The ion spray voltage for positive and negative ion modes was + 5500 and – 4500 V respectively. Other, mass parameters for the positive and negative ion modes were as follows: source temperature was 450 $^\circ\text{C}$, curtain gas was 25 psig, nebulizer gas was 50 psig and turbo gas was 50 psig. Full scan spectra were recorded in the mass

range of m/z 50–1000. The product ion spectra of the selected ion were obtained by the collision energy between 20 and 30 eV for positive ion mode and – 20 and – 30 eV for the negative ion mode. Instrument operation and data analysis were performed using Applied Biosystems Analysts 1.5 software.

Analytical condition for GC-MS

For GC-MS, an Agilent 7000 C GC-MS triple quadrupole mass analyzer (Agilent technologies, USA) combined with an Agilent 7890B GC system was used. The GC includes an MPS (multipurpose sampler) autosampler (Gerstel, Germany), chromatographic columns, a column oven, a syringe injector port, a vaporization chamber, a carrier gas controller and a pressure-controlled tee (PCT) backflushing system based on a purged union. Two columns were installed in the GC system: the original HP-5MS UI, a 30 m $\times$ 0.250 mm $\times$ 0.25 μm capillary column (Agilent technologies), was cut into 5 m $\times$ 0.250 mm $\times$ 0.25 μm (column 1) and 15 m $\times$ 0.250 mm i.d. $\times$ 0.25 μm (Column 2). Column 1 is a precolumn, connected with the inlet on one end of the purged union, and analytical column 2, was set between the other end of the purged union and the MS source (Katerina and Philip 2012). Instrument control and data processing were performed by Agilent MassHunter Workstation software, and the autosampler was controlled by Gaerstel Maestro 1 software.

Sample injection conditions were as follows: programmable temperature vaporizer (PTV) injection solvent vent mode (25 ml/min vent flow, 5 psi vent pressure, 0.3 min vent time), inlet pressure 5.6 psi, injection volume 5 μL , septum purge flow 3 ml/min, and purge flow to split vent 50 ml/min at 1.5 min.

Helium was used as the carrier gas. The flow was set accordingly: (1) column 1: 1.1 ml/min for 15.8 min, ramp 100 ml/min to 2.5 ml/min (hold 2.68 min), and ramp 100 ml/min to 5 ml/min (2) column 2: 1.2 ml/min for 18.6 min and, ramp 100 ml/min to 2.4 ml/min. Oven temperature was programmed as follows: 1.5 min at 60 $^\circ\text{C}$, ramp 50 $^\circ\text{C}/\text{min}$ to 150 $^\circ\text{C}$ (hold 0 min), 8 $^\circ\text{C}/\text{min}$ to 240 $^\circ\text{C}$ (hold 0 min), 50 $^\circ\text{C}/\text{min}$ to 280 $^\circ\text{C}$ (hold 2.5 min), and 100 $^\circ\text{C}/\text{min}$ to 290 $^\circ\text{C}$ (hold 2.05 min). The ionization voltage was – 70 eV and the source temperature was 280 $^\circ\text{C}$. Post-run temperature, post-run time and solvent delay time were 300 $^\circ\text{C}$, 3 min and 4 min, respectively.

Analytical condition for UPLC-DAD

A waters Acquity UPLC-DAD (Milford, MA, USA) system was employed using an Acquity BEH C18 chromatographic column (2.1 $\times$ 100 mm, 1.7 μm). The chromatographic separation was investigated using mobile

phase A composed of 50:22.5:22.5:5 water + 5 mM ammonium formate:methanol:acetonitrile:ethyl acetate and mobile phase B composed of 50:50 acetonitrile:ethyl acetate. The mobile phase flow rate was 0.2 ml/min and column oven temperature was 40 °C. The gradient flow was 22% B at 0 min, was ramped up to 85% over the course of 15 min, followed by a linear gradient to 99% up to 35 min, then reached 22% up to 38 min and was kept unchanged until 45 min. The injection volume was 10 μ L.

Results and discussion

Matrix component identification by the LC–ESI–MS and LC–ESI–MS/MS positive ion mode

LC–MS/MS has been extensively applied in the last few decades in plant metabolomics profiling because it is powerful and one of the most sophisticated techniques for identify trace components. Representative total ion chromatograms (TIC) were obtained in the LC–ESI–MS positive ion mode for the both methods, AOAC and CEN (Fig. 1a, b). In this study, six component peaks were detected in the positive ion mode. The fragmentation patterns are shown (Supplementary Fig. 1) and the results are summarized in Table 1. Component (1) gave a molecular ion $[M+H]^+$ at m/z 481, which was proposed as beta-ecdysone. The fragments at m/z 445 $[M+H-2H_2O]^+$ and 427 $[M+H-3H_2O]^+$ were produced by the sequential losses of two and three molecules of water respectively (Jocelyne et al. 1994; Daniele et al. 2014). The ion m/z 371 was generated due to loss of the $CH_2=C(CH_3)_2$ moiety from $[M+H-3H_2O]^+$ (m/z 427), corresponding to C24 and C25 cleavage (Jocelyne et al. 1994). A similar molecular ion $[M+H]^+$ at m/z 481 with identical fragments was also observed, component (2), which could be the isomer of beta-ecdysone. Component (3) showed the molecular ion $[M+H]^+$ at m/z 675 and its MS/MS spectrum yielded a typical fragment ion at m/z 347 $[M+H-328]^+$, which was consistent with spinacetin (Mi et al. 2008). Two other fragment ions of m/z 293 and 275 could be produced by the successive loss of $3H_2O$ and $4H_2O$ from $[M+H-328]^+$ (m/z 347), respectively; this component might be a spinacetin derivative. Component (4) presented a molecular ion peak $[M+H]^+$ at m/z 277. The fragment ion observed at m/z 135, 107, and 93 was similar to the MS/MS observation of 14, 15-dehydrocrepenynic acid (Wei et al. 2014); hence it was identified as this compound. Mass spectra in ESI–MS presented a molecular ion at m/z 564 which corresponds to $[2M+2H]^+$. Following MS/MS fragmentation of $[2M+2H]^+$ (m/z 564), two species of product ions were introduced at m/z 282 $[2M+2H-282]^+$ and 247 $[2M+2H-282-35]^+$. The characteristic mass ion m/z at 247 indicates

the neutral loss of ammonia (17 units) and water (18 units) from m/z 282. This phenomenon was matched with characteristics of the component (5) dimer of cis-9, 10-octadecenoamide. Component (6) showed the molecular ion $[M+H]^+$ in electrospray ionization at m/z 338. The characteristic fragment ions at m/z 321 $[M+H-NH_3]^+$ and 303 $[M+H-NH_3-H_2O]^+$ were likely generated from the loss of one molecule of ammonia (17 units) followed by a water molecule (35 units). The fragmentation was in agreement with cis-13, 14-docosenamide (Benjamin et al. 1995).

Matrix component identification by the LC–ESI–MS and LC–ESI–MS/MS negative ion mode

The LC–ESI–MS full scan in the negative ion mode for the QuEChERS AOAC and CEN method is shown in Fig. 1c, d, respectively. The fragmentation patterns are presented in Fig. 2 of the Supplementary Material, which are summarized in Table 1. Eight components were identified in the negative ion mode. Component (7) exhibited the deprotonated molecular ion $[M-H]^-$ at m/z 327. The major fragment ions were observed at m/z 309, 291, 229 and 211, which is characteristic of an oxylipin compound, oxo-dihydroxy-octadecenoic acid (DHODE) (Laura et al. 2014; Eulogio et al. 2015). Component (8) presented a molecular ion $[M-H]^-$ at m/z 345. In MS/MS, two predominant fragment ions at m/z 330 and 315 were generated by the consecutive losses of two methyl groups. Thus, the component contains two methyl groups. Another minor ion, m/z 287 $[M-H-2CH_3-CO]^-$, was produced after losses of $2CH_3$ and CO. Hence, this component was identified as spinacetin (Ulla 2001). Component (9) yielded the deprotonated molecular ion $[M-H]^-$ at 329, which lost one H_2O molecule to produce fragment ion m/z 311. Two other fragment ions at m/z 229 and 211 were tentatively assigned as originating from 329 and 311, respectively, after loss of the $[HO-CH=CH(CH_2)_3CH_3]$ moiety (100 units), a characteristic side chain of the oxylipin molecule. The presence of fragment ion m/z 171 $[CHO(CH_2)_7COO]^-$ indicates the breakdown of 58 $[-CH_2CH(OH)CH_2-]$ units from m/z 229 $[COO(CH_2)_{10}COOH]^-$, which is common in the fragmentation of many linolenic acid oxidation products. Based on this information component (9) could be identified as 9,10,13-trihydroxy-11-octadecenoic acid (THODE) (Tonu et al. 2009). Component (10) showed a similar deprotonated molecular ion as component (9), $[M-H]^-$ m/z 329, but differed in mass fragmentation patterns, indicating their different molecular characteristics. This was followed by the MS/MS major fragment ions at m/z 314, 299, 271 and 227, which resembled triclin (Joaquim et al. 2007; Laura et al. 2012). Component (11) led to a deprotonated molecular ion $[M-H]^-$ at m/z 359, and it underwent two consecutively methyl losses to establish the

Fig. 1 Total ion chromatogram (TIC) of spinach following QuEChERS sample preparation obtained by the LC–ESI–MS positive ion mode: **a** AOAC method and **b** CEN method; and LC–ESI–MS negative ion mode: **c** AOAC method and **d** CEN method

major fragments at m/z 344 and 329. Other minor fragments were obtained at m/z 301. This was concurrent with jaceidin (Ulla 2001). Component (12) presented the molecular ion $[M-H]^-$ at m/z 311. The fragment ion at m/z 293 corresponded to eicosanoic acid, showing a loss of a water molecule, reported previously in the literature (Jinfeng et al. 2017), in which component (12) should be eicosanoic acid. Component (13) presented a molecular ion at m/z 293. Due to the loss of one water molecule, it produced m/z 275, the base peak, which was previously described as 9-hydroxy-octadecatrienoic acid (Tobias et al. 2009). Component (14) gave the molecular ion $[M-H]^-$ at m/z 609. The component eliminates 294 units, which is the

characteristic mass of a hexose (162 units) and a pentose (132 units) moiety, and produced m/z 315; therefore, it is a species of isorhamnetin. Two fragment ions at m/z 293 and 275 arose from the liberation of two water molecule consecutively from m/z 315. The component was verified as isorhamnetin-O-hexoside-pentoside (Filipa et al. 2017).

Matrix component identification in QuEChERS extract by GC–MS

The remaining components in spinach were investigated by GC–MS after AOAC and CEN sample preparations. The

Table 1 LC–ESI–MS/MS identification of matrix components in spinach following QuEChERS AOAC or CEN sample preparation method

Components No.	Ionization	Precursor ion	Product ion	Tentative assignment	Nature
1	[M+H] ⁺	481	445, 427, 409, 371, 165	beta-ecdysone	Phytosteroid
2	[M+H] ⁺	481	445, 427, 371, 165	beta-ecdysone	Phytosteroid
3	[M+H] ⁺	675	347, 293, 275	spinacetin derivative	Falvonoid
4	[M+H] ⁺	277	135, 107, 93	14, 15-dehydrocrepenynic acid	Fatty acid
5	[2M+2H] ⁺	564	282, 247	dimer of cis-9, 10-octadecenoamide	Fatty acid amide
6	[M+H] ⁺	338	321, 303	cis-13, 14-docosenamide	Fatty acid amide
7	[M-H] ⁻	327	309, 291, 229, 211	oxo-dihydroxy-octadecenoic acid, (DHODE)	Fatty acid
8	[M-H] ⁻	345	330, 315, 287	spinacetin	Falvonoid
9	[M-H] ⁻	329	229, 211	9,10,13-trihydroxy-11-octadecenoic acid (THODE)	Fatty acid
10	[M-H] ⁻	329	314, 299, 271, 227	tricin	Falvonoid
11	[M-H] ⁻	359	344, 329, 301	jaceidin	Falvonoid
12	[M-H] ⁻	311	293	eicosanoic acid	Fatty acid
13	[M-H] ⁻	293	275	9-hydroxy-octadecatrienoic acid	Fatty acid
14	[M-H] ⁻	609	315, 293, 275	isorhamnetin-O-hexoside-pentoside	Falvonoid

peak integration was done using Agilent MassHunter Workstation version B.02.00 software. In Fig. 2, the TIC of the AOAC and CEN method is shown. The peaks corresponding to their mass spectrum were compared with the data set in the National Institute of Standards and Technology (NIST, U.S.) library, version 2.0, for interpretation.

Following the AOAC and CEN sample preparations the total number of molecular ion peaks was identified to be fifty and fifty-seven peaks respectively in GC–MS. The possible components with representative retention time (t_r), compound identity, compound nature, peak area (%) and molecular formula (M. F.) are listed in Table 1 for AOAC

Fig. 2 Total ion chromatogram (TIC) of spinach obtained by GC–MS for the QuEChERS: **a** AOAC method and **b** CEN method

and Table 2 for CEN of Supplementary Material. During sample preparation matrix components were not derivatized; therefore, it could be anticipated that the identified components in GC–MS enter through the column with pesticides residues, which may significantly cause a matrix enhancement effect. Matrix components were distributed from 4 to 20 min in the column, which indicates components being eluted with wide characteristics. These components can be classified as different groups such as fatty acids, fatty acid esters, and terpenoids. Eight major components were found to be present AOAC extract: 7,10,13-hexadecatrienoic acid, methyl ester (10.55%); n-hexadecanoic acid (9.62%); 9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-(11.17%); 9,12-octadecadienoic acid (Z,Z)-, methyl ester (9.54%); phytol (5.29%); 9,12-octadecadienoic acid (Z,Z)-(9.29%); methyl 5,9-heptadecadienoate (21.47%) and 9,12,15-octadecatrienoic acid, (Z,Z,Z)-(4.06%), which constituted 80.99% of the total area of all components in the AOAC method. Eight major components were found to be present CEN extract: 7,10,13-hexadecatrienoic acid, methyl ester (9.93%); n-hexadecanoic acid (7.39%); 9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-(9.37%); 9,12-octadecadienoic acid (Z,Z)-, methyl ester (11.94%); phytol (5.02%); 9,12-octadecadienoic acid (Z,Z)-(11.8%); methyl 5,9-heptadecadienoate (10.36%) and 9,12,15-octadecatrienoic acid, (Z,Z,Z)-(6.15%), which constituted 71.96% of the total area of all components in the CEN method.

Matrix component identification by UPLC-DAD

HPLC–DAD was employed in the separation and identification of pigments in spinach. Identification of unknown components was assisted by characteristic absorption spectra via comparison with standard retention time (t_r), maximum absorbance spectra, and published literature data. The chromatogram profiles of pigments for the standard and, AOAC and CEN method are shown in Fig. 3. Investigation of eluents at 440 nm revealed that samples consist of various kinds of carotenoids and chlorophylls (Vasco et al. 2014). Tocopherol shows a maximum absorbance at 290 nm (Jennifer et al. 2003). Pigment elution time was recorded in the following order: neoxanthin (t_r 5.70 min), violaxanthin (t_r 6.18 min), antheraxanthin (t_r 7.50 min), zeaxanthin (t_r 8.82 min), lutein (t_r 9.04 min), chlorophyll *b* (t_r 15.76 min), gamma-tocopherol (t_r 16.06 min), alpha-cryptoxanthin (t_r 16.07 min), beta-cryptoxanthin (t_r 16.20 min), alpha-tocopherol (t_r 16.96 min), chlorophyll *a* (t_r 17.01 min), pheophytin *a* (t_r 20.48 min), alpha carotene (21.80 min) and beta-carotene (t_r 22.37 min). Three components were identified in the AOAC extracts (neoxanthin, violaxanthin and lutein). Eight component (neoxanthin, violaxanthin, zeaxanthin,

lutein, chlorophyll *b*, chlorophyll *a*, pheophytin *a* and beta-carotene) peaks were identified in the CEN extract. The characteristics wavelength for maximum absorbance (λ_{max}) of identified or unidentified pigments are shown in Supplementary Material Table 3.

Comparison of matrix components between the AOAC and CEN methods

The AOAC and CEN methods showed very similar chromatogram peaks in LC and GC with little differences in intensity (data not shown). The matrix component amounts differed between the AOAC and CEN methods, but this difference was small, except for pigment contents by the use of different amounts of GCB. By UPLC-DAD in the AOAC method only three peaks were identified; neoxanthin, violaxanthin and lutein.

Conclusion

In this study, a detail investigation of spinach matrix components by QuEChERS AOAC and CEN sample preparation methods is presented. To our knowledge, this is the first attempt to identify various kinds of matrix components by thorough investigation applying three different instruments, LC–ESI–MS/MS, GC–MS and UPLC-DAD. Using LC–ESI–MS/MS, fourteen components were identified; all components were identified for the first time in the AOAC and CEN extract of spinach. Most of the components in LC–ESI–MS/MS were found to be flavonoids and fatty acids. Fifty and Fifty-seven ion peaks were detected via the AOAC and CEN method respectively by GC–MS. Among them, eight were major components, such as 7,10,13-hexadecatrienoic acid, methyl ester; n-hexadecanoic acid; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; phytol; 9,12,15-octadecatrienoic acid, (Z,Z,Z)-; 9,12-octadecadienoic acid (Z,Z)-; 9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- and methyl 5,9-heptadecadienoate. Additionally, three carotenoids were identified in AOAC extract, and six carotenoids and two chlorophylls were identified in the CEN extract. The AOAC sample preparation method showed similar kinds of matrix components compared to that of the CEN method in LC and GC. The AOAC method revealed less matrix components than the CEN method; however the amount of matrix components was not much different between the AOAC than CEN methods, except for the pigment contents by the use of different amounts of GCB.

The matrix of spinach after QuEChERS cleanup was identified as having many components, flavonoids, fatty acids, fatty acids esters, terpenoids, carotenoids and chlorophylls, which shows the need for more cleanup. PSA

Fig. 3 Comparison of chromatograms obtained for pigment identification and characterization of spinach extracts using UPLC-DAD set at 440 nm for carotenoid and chlorophyll detection and 290 nm for tocopherol detection: **a** standard pigments in solution, **b** pigment in AOAC extract, and **c** pigment in CEN extract

and GCB sorbents are used in QuEChERS sample preparation frequently, and knowledge of matrix components relating to sorbent adsorption is necessary for method development and improvement. Moreover, understanding of matrix component behavior would assist in the assumption of matrix components of other commodities and, hence lead to choosing a better method for pesticide residue analysis. In addition, High Resolution Mass Spectrometry would be a very helpful tool for these type studies, giving almost a total fingerprint of the matrix.

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