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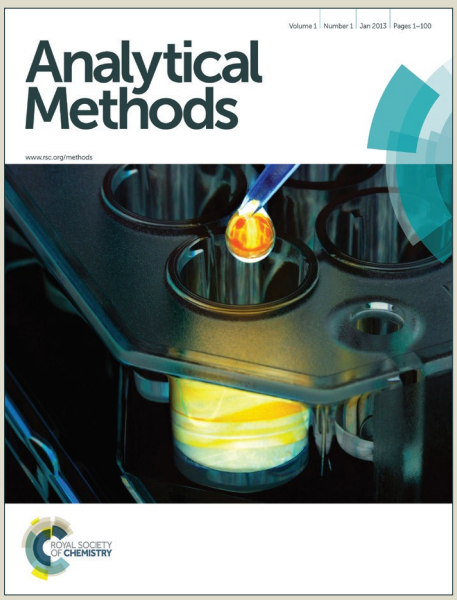

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ARTICLE

A facile method for simultaneous analysis of phytosterols, erythrodiol, uvaol, tocopherols and lutein in olive oils by LC-MS

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In this work a facile, sensitive and reliable method was developed and validated for simultaneous analysis of phytosterols, erythrodiol, uvaol, tocopherols and lutein in olive oils. Analytes extracted from saponified olive oils were directly determined by liquid chromatography-mass spectrometry (LC-MS) with atmospheric pressure chemical ionization (APCI). L-ascorbic acid sodium salt was added into oils as antioxidant prior to the saponification process, since tocopherols are sensitive to the effect of light and elevated temperature. We found that using L-ascorbic acid sodium salt made the sample treatment become a facile procedure. In this case we no longer need to use amber glass tubes or enwrap the tubes with aluminum foil. Separation was achieved on a C18 column with a gradient of acetonitrile/water (0.1% formic acid). Erythrodiol and uvaol were quantified separately for the first time in HPLC based analysis. The selected ion monitoring (SIM) mass spectra of 14 analytes were measured by mass spectrometer with APCI ion source. The parameters including the linearity of the standard curves, the detection and quantification limits, recovery, repeatability and reproducibility were evaluated for the validation of the method.

1 Introduction

The unsaponifiables of olive oils is composed of many minor constituents including phytosterols, triterpene dialcohols, tocopherols and xanthophylls (including lutein). Although this fraction represents only 1-2% of the whole, these compounds play an important role in olive oils and have attracted great interest in the past few years[1]. On the one hand, these compounds act as functional components in olive oils with potential health benefits. Phytosterols can decrease the serum cholesterol levels, and hence protect human against cardiovascular disease[2]. Tocopherols act as free radicals scavengers and prevent the propagation of lipid peroxidation in olive oils and biological systems[3]. Lutein can protect the eyes from the damages of the high-energy photons of blue light and oxidative stress. On the other hand, the unsaponifiable fraction has many applications in the detection of extra virgin olive oil adulteration[4]. These compounds, especially phytosterols, could be used as fraud tracers in virgin olive oils[5, 6]. However, the levels of these compounds are known to vary among olive species, growing conditions, extraction and refining procedures[7]. Furthermore, phytosterols undergo oxidation during storage, and the appearance of tocopherols may reduce the oxidation rate[8, 9]. In order to better understand the unsaponifiable fraction qualitatively and quantitatively in olive oils, analytical method allowing multiple components quantification in a single procedure would therefore be preferred.

Traditionally, sterols and triterpene dialcohols in olive oils are analyzed by the use of gas chromatography (GC) [10, 11]. Briefly, the unsaponifiable fraction of the olive oils is extracted with diethyl ether and washed with water. The phytosterols in the unsaponifiable fraction are separated by thin-layer chromatography (TLC) on silica gel plates[10] or solid-phase extraction[11] and derivatized prior to GC analysis. This method is laborious, time-consuming and cannot analyze the four classes of unsaponifiable fraction of olive oils, i.e. phytosterols, triterpene dialcohols, tocopherols and xanthophylls in a single procedure.

High performance liquid chromatography (HPLC) with different detectors has been proved to be an effective alternative compared with GC for determination of phytosterols in foods. M. Slavin et al.[12] successfully quantified lutein, 3 phytosterols and 3 tocopherols in soybeans by HPLC coupled to evaporative light scattering detector (ELSD) and UV/Vis absorbance detector. M. M. Delgado-Zamarreño et al.[13] demonstrated that 3 tocopherols and 4 phytosterols in seeds and nuts could be simultaneously analyzed by HPLC coupled to diode array detection (DAD). Baseline separation of peaks should be achieved in order to quantify chemicals in a HPLC experiment. However, due to the chemical structure similarity of phytosterols, it is hard to achieve baseline separation for all of the analytes in a multi-component analysis. In M. Slavin's work[12], baseline resolution was not achieved for campesterol, δ-tocopherol and stigmasterol. Campesterol and stigmasterol were also not separated in M. M. Delgado-Zamarreño's work[13]. Furthermore, ELSD as well as DAD have a fairly low sensitivity compared to other detectors such as MS

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detection. Therefore, HPLC coupled to ELSD or DAD is not an ideal method for analyzing phytosterols and tocopherols due to the low selectivity and sensitivity.

As reported by Cañabate et al.[14], liquid chromatography-mass spectrometry (LC-MS) with atmospheric pressure chemical ionization (APCI) was used for the first time to identify and quantify seven phytosterols in olive oils. Baseline separation of peaks is not necessary because of the high selectivity of MS detection; LC-MS can therefore be used to analyze more components in a single procedure. This pioneering work provided a much simpler way than the official method, which involves saponification, extraction, TLC, and derivatisation. From then on, many papers were published on determination of phytosterols and/or triterpene dialcohols in olive oils by LC-MS with APCI[14-18]. Zarrouk et al.[19] developed a LC-MS method for simultaneous analysis of sterols, tocopherols and triterpene dialcohols in vegetable oils, and enabled a great number of compounds to be quantified in a single procedure. It is well recognized that test samples exposed to light and elevated temperature would cause accelerated degradation of tocopherols and xanthophylls[12]. In order to prevent the analytes from light, glass tubes used in experiments need to be enwrapped with aluminum foil, which is laborious and time-consuming. Besides, in all of the above LC-MS methods, the erythrodiol and uvaol had not been separated chromatographically and therefore had not been quantified separately, since the target ion of the two analytes are the same[14, 16, 17, 19].

To the best of our knowledge, there is no reference in which simultaneous analysis of phytosterols, erythrodiol, uvaol, tocopherols and xanthophylls in olive oils has been carried out. Only a few references could analyze three classes of the unsaponifiable fraction of olive oil in a single procedure, but some drawbacks were revealed, such as low selectivity and sensitivity[12, 13], the erythrodiol and uvaol cannot be quantified separately[14, 16, 17, 19] and xanthophylls are not included in the analysis[16, 18, 19], etc. The present work therefore reports for the first time the simultaneous analysis of phytosterols, erythrodiol, uvaol, tocopherols and lutein in olive oils by LC-MS.

2 Materials and methods

2.1 Materials

β-sitosterol (97.8%), cholesterol (86.3%), stigmasterol (89.0%), fucosterol (11.2%), brassicasterol (92.5%), ergosterol (94.6%), erythrodiol (96.6%), uvaol (97.0%), α-tocopherol (92.8%), β-tocopherol (93.4%), γ-tocopherol (98.3%), δ-tocopherol (97.1%) and lutein (90.0%) were purchased from Chromadex (Irvine, CA). Campesterol (65.0%) was from Sigma-Aldrich (St. Louis, MO). HPLC-grade ethanol, methanol, acetonitrile and *n*-hexane were from Honeywell (Ulsan, Korea). Formic acid was from Fisher Scientific (Pittsburgh, PA). KOH, anhydrous sodium sulphate and L-ascorbic acid sodium salt were from Beijing Shiji (Beijing, China). Milli-Q water of 18.2 MΩ·cm^{-1} resistivity was used throughout (Millipore, Billerica, MA).

Stock solutions (1.0 mg mL^{-1}) of the individual compounds were dissolved in methanol and stored in the dark at -18 °C for at least 2 months. The multi-compounds working standard solutions were prepared daily by appropriate dilution of the stock solution with acetonitrile.

2.2 Sample preparation

Extra virgin olive oil samples coming from Antequera area of Spain (harvested in 2013, bottled by COFCO corporation of China) were purchased from supermarket in 2014 and stored at 4 °C in the dark before analysis. The method for the saponification, the separation of analytes from the olive oil was modified from the published method developed by Martínez-Vidal et al.[17].

Briefly, samples (100 mg olive oil) were accurately weighed into the 10 mL screw-capped test tubes. 0.25 mL of L-ascorbic acid sodium aqueous solution (0.2 g mL^{-1}) and 2 mL of KOH ethanolic solution (2 M) were added and vortexed. The tubes were then introduced to a water bath and heated at 60 °C for 1 h. After cooling at room temperature, 2 mL water and 2 mL *n*-hexane were added. Mixtures were vortexed vigorously for 1 min then centrifuged (Thermo Fisher Scientific, MA) for 10 min at 1600 ×*g* and the supernatant was transferred to a new tube. The extraction was repeated for two times and the *n*-hexane fractions were combined (totally 6 mL). Finally, the *n*-hexane extracts were washed with water until the wash water gave a neutral reaction and then dried with anhydrous sodium sulphate. 3 mL of the extracts were placed in a new tube and the solvent was evaporated under nitrogen. The residue was re-dissolved in 1.0 mL acetonitrile and filtered through 0.22 μm PTFE syringe filters prior to LC-MS analysis.

2.3 HPLC separation

Separation of analytes was carried out on Agilent 1200 HPLC system with an Agilent ZORBAX Eclipse XDB-C_{18} column (5 μm, 4.6 mm×150 mm). The column temperature was set to 30 °C and the injection volume was 10 μL. The analytes were separated with a gradient mobile phase at a flow rate of 0.8 mL min^{-1}. The mobile phase were water (0.1% formic acid) (Phase A), acetonitrile (Phase B) and methanol (Phase D), and the gradient program was: from 0 to 8 min, held on 2% A:95% B:3% D; from 8 to 10 min, 2% A:95% B:3% D to 2% A:0% B:98% D; from 10 to 28 min, held on 2% A:0% B:98% D; from 28 to 30 min, 2% A:0% B:98% D to 2% A:95% B:3% D; from 30 to 35 min, held on 2% A:95% B:3% D.

2.4 MS detection

The HPLC system was coupled to an Agilent 6410B mass spectrometer equipped with an APCI interface operating in positive ion mode. The optimized temperature and flow rate of the nitrogen dry gas were 350 °C and 6 L min^{-1}, respectively. The vaporizer temperature was 350 °C and the nebulizer gas pressure was 60 psi. The capillary voltage was +3500 V and the corona discharge current was 5 μA. Detection was performed in selected ion monitoring (SIM) mode. Analytes identification was based on the comparison of their retention times with those of authentic standards. Identification of peak was also done with spiked samples at different concentration levels. The molecular mass, observed ions and retention time (RT) for standards were listed in Table 1. Chemical structures and APCI-MS spectra of the analytes were provided in the Electronic Supplementary Information (ESI).

Table 1 Molecular weight, observed ions and RT for standards analyzed by LC-MS

Analytes	RT (min)	Molecular weight	$[M+H-H_2O]^+$	$[M+H]^+$
Erythrodiol	6.7	442.7	425.3	
Lutein	6.8	568.9	551.4	
Uvaol	7.1	442.7	425.3	
δ-tocopherol	14.6	402.7		403.3
β/γ-tocopherol	16.3	416.7		417.3
Ergosterol	17.1	396.7	379.3	
α-tocopherol	18.4	430.7		431.3
Brassicasterol	19.6	398.7	381.3	
Cholesterol	20.2	386.7	369.3	
Fucosterol	20.5	412.7	395.3	
Campesterol	22.3	400.7	383.3	
Stigmasterol	22.4	412.7	395.3	
β-sitosterol	24.7	414.7	397.3	

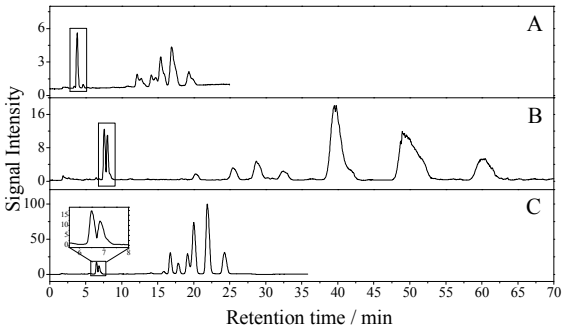

Fig. 1 LC-MS chromatograms of a standard mixture of phytosterols, triterpene dialcohols, tocopherols and lutein eluted with different mobile phase, A: water (0.1 % formic acid)/methanol (2:98); B: water (0.1 % formic acid)/acetonitrile (2:98); C: a gradient of water (0.1 % formic acid)/acetonitrile/methanol mixture as described in section 2.3. Eluent flow rate 0.8 mL min^{-1}. The EIC of erythrodiol and uvaol was enlarged and shown in the insets.

The analysis of the extracts was carried out under the optimum instrument conditions described above. Calibration curves for every analytes were obtained from the multi-compounds working standard solutions at different concentration levels, selected as representative of the range of concentrations in the extra virgin olive oil samples. Linear calibration curves were constructed by least-squares regression of concentration versus peak area of the calibration standards. Quantification of the analytes was carried out using the extracted ion chromatograms (EIC) at *m/z* 425.3, 551.4, 403.3, 417.3, 379.3, 431.3, 381.3, 369.3, 395.3, 383.3 and 397.3. Erythrodiol, uvaol, lutein, δ-tocopherol, ergosterol, α-tocopherol, brassicasterol, cholesterol, fucosterol, campesterol, stigmasterol and β-sitosterol were quantified individually in mg kg^{-1}, while β- and γ-tocopherols were quantified together because they had the same retention time and provided the same *m/z* in MS.

3 Results and discussion

3.1 Optimization of HPLC separation

The separation of the four classes of compounds was optimized in terms of column selection and mobile phase composition. A test mixture of 14 standards (0.4 μg mL^{-1} for erythrodiol, uvaol, lutein, stigmasterol, β-tocopherol, γ-tocopherol, δ-tocopherol, brassicasterol, cholesterol, ergosterol and fucosterol; 1.0 μg mL^{-1} for campesterol; 4.0 μg mL^{-1} for α-tocopherol and 40 μg mL^{-1} for β-sitosterol) was used in the experiment.

3.1.1 Column selection

Reversed phase C_{18} column has been successfully used to separate phytosterols, triterpene dialcohols in HPLC-MS detection[14, 19]. α-, γ- and δ-tocopherols have also been successfully quantified on C_{18} columns[20], and C_{30} columns offer the extra ability to separate the β- and δ-tocopherols. Olive oils contain only minimal β-tocopherol, its co-elution with γ-tocopherol is therefore not generally considered a problem in olive oil analysis. Besides, C_{18} is one of the most widely used columns in HPLC experiments. Taken together, two columns with C_{18} stationary phase were therefore tested: Agilent ZORBAX Eclipse XDB-C_{18} 4.6 mm×150 mm i.d., 5 μm and Agilent ZORBAX Eclipse XDB-C_{18} 4.6 mm×250 mm i.d., 5 μm. To compare the performances of columns, the test mixture mentioned above was injected and eluted with water (0.1 % formic acid)/acetonitrile (2:98, v/v). We found that the longer column gave a better separation of the aforementioned standard mixture. For the 14 analytes, especially the phytosterols, adequate separation is not easily achieved. Fortunately, in HPLC-MS baseline separation of peaks is not mandatory because of high specificity and selectivity of the detection method. Therefore, the shorter column also met our expectation, since the analytes with the same quantitative ion (except for β- and γ-tocopherols) were successfully separated with both two columns. On the other hand, separation of analytes was achieved in 55 min using short column, while 100 min were necessary to do the same with the longer column. Therefore, the shorter column, i.e. Agilent ZORBAX Eclipse XDB-C_{18} 4.6 mm×150 mm i.d., 5 μm was more suitable for this work.

3.1.2 Optimization of mobile phase

The mobile phase composition was optimized to achieve effective separation of analytes. As the most used mobile phase solvent in HPLC-MS analysis, methanol and acetonitrile were evaluated in our test. The test mixture of 14 standards was injected and eluted by two kinds of mobile phases: water (0.1 % formic acid)/methanol (2:98) and water (0.1 % formic acid)/acetonitrile (2:98), respectively. As shown in Fig. 1, we found that the analytes were eluted within 21 min with mobile phase of water/methanol mixture, while 62 min were necessary with water/acetonitrile. Besides, the peak shapes of analytes eluted by water/methanol mixture were much sharper than those eluted by water/acetonitrile. Considering the efficiency of analysis, mixture of water/methanol was preferred.

Although baseline separation of peaks is not mandatory in HPLC-MS analysis, chromatographic separation of analytes that have the same quantitative ion is still necessary. Except for β-tocopherol and γ-tocopherol, erythrodiol and uvaol have the same quantitative ion of *m/z* 425.2, and *m/z* 395.5 for fucosterol and stigmasterol. In order to quantify every analyte separately, both of the two pairs should be separated chromatographically. However, erythrodiol and uvaol could not be separated by mobile phase of water (0.1 % formic acid)/methanol (2:98) as indicated in Fig. 1A. In order to separate the two analytes, as well as maintain the sharp peak shape and short analysis time, a gradient elution as described in section 2.3 was developed. As indicated in Fig. 1C, we successfully separated erythrodiol and uvaol by gradient elution for the first time in HPLC experiments. Meanwhile, the separation of analytes was achieved only in 26 min and the peak shape was still relatively sharp. Therefore, a gradient elution of water (0.1 % formic acid)/acetonitrile/methanol mixture as described in section 2.3 was used in this work.

3.2 MS determination

Phytosterols, erythrodiol and uvaol are highly lipophilic with few polar groups and are hard to ionize by electrospray ionization (ESI) methods, so APCI with positive ion mode must be used instead of ESI in the analysis[14]. Besides, recent studies have demonstrated that APCI with positive ion mode was suitable for the determination of tocopherols[19] and lutein[21]. Therefore, APCI with positive ion mode was used for quantifying phytosterols, erythrodiol, uvaol, tocopherols and lutein in our work. Detection was performed in SIM mode, and the monitoring ions of the analytes were *m/z* 425.2 (for erythrodiol and uvaol), 551.7 (for lutein), 402.2 (for δ- tocopherol), 416.2 (for β and γ-tocopherol), 379.5 (for ergosterol), 430.2 (for α-tocopherol), 381.2 (for brassicasterol), 369.7 (for cholesterol), 395.5 (for fucosterol and stigmasterol), 383.4 (for campesterol) and 397.5 (for β-sitosterol).

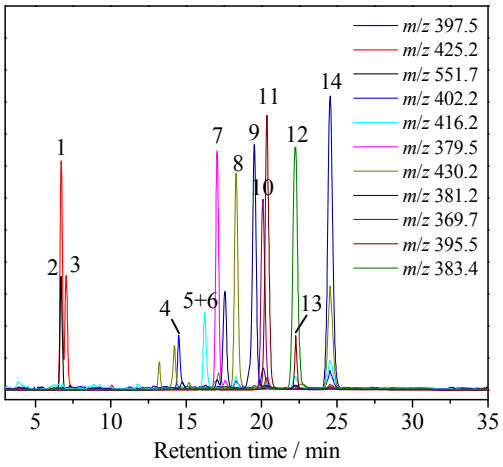

Fig. 2 Extracted ion chromatograms of the mixture of 14 analytes. Peaks number from 1 to 14 correspond to erythrodiol, lutein, uvaol, δ-tocopherol, (β+γ)-tocopherols, ergosterol, α-tocopherol, brassicasterol, cholesterol, fucosterol, campesterol, stigmasterol and β-sitosterol, respectively.

Some of the APCI source parameters (nebulizer gas pressure, capillary voltage and corona discharge current) were adopted from the literatures[19] while others (vaporizer temperature, flow rate and temperature of the dry gas) were optimized by direct infusion experiments with the test mixture of 14 standards to achieve the best conditions regarding peak intensity and resolution. The optimized values could be found in section 2.4. The transfer parameter, i.e. the fragmentor of each analyte was optimized by direct infusion experiments with the individual standards, and the optimized fragmentor values for each analyte were listed in Table 1. The EIC of the mixture of 14 analytes is shown in Fig. 2.

3.3 Optimization of sample treatment

Saponification of the oil matrix was necessary in sample preparation of phytosterols, erythrodiol and uvaol, because triacylglycerol should be removed before analysis. However, test samples exposure to light and elevated temperature caused the accelerated degradation of tocopherols and xanthophylls. Special precautions therefore should be taken in the sample treatment procedures.

The saponification could be performed by refluxing[14], at 60-80 °C[17, 19], or room temperature[22]. Cold saponification was avoided in our work because it was time consuming (usually over night). In order to minimize the risk of degradation, a saponification temperature of 60 °C was chosen and the reaction time was 1 hour, which have been demonstrated a suitable time for olive oil[17].

The effect of light on the analysis of unsaponifiable fraction was also evaluated in our work. Olive oil samples that spiked with a mixture of standards mentioned in section 3.1 were treated under two different conditions and analyzed by LC-MS: one with light and the other protected from light. The protection of analytes from light was achieved by enwrapping the glass tubes with aluminum foil. As shown in Fig. 3, the content of tocopherols and lutein differed significantly between the two sample treatment methods. Compared with the sample protected from light, the content of α-tocopherol, (β+γ)-tocopherols, δ-tocopherol and lutein decreased 50.7 %, 23.9 %, 51.1 % and 25.4 % in the sample treated with light, respectively. Therefore, it is crucial to protect the analytes from light during the sample treatment process. However, it is laborious and time-consuming to enwrap the glass tubes with aluminum foil every time before experiment. Amber glass tubes can protect the analytes from light during sample treatment, but this kind of glass tube is quite expensive and is not commercial available in China.

It was demonstrated that the addition of antioxidant could protect the analytes from degradation during sample treatment[12]. In our work, we tried to use L-ascorbic acid sodium salt as an antioxidant, which is more environmental friendly compared with tert-butyl hydroquinone (TBHQ). 0.25 mL of L-ascorbic acid sodium aqueous solution (0.2 g mL^{-1}) was added into each of two aforementioned spiked olive oil samples, and then treated with two different conditions and analyzed by LC-MS: one with light and the other protected from light. As shown in Fig. 3, the content of analytes was almost the same for the two samples, indicating that the addition of L-ascorbic acid sodium solution could protect α-tocopherol, β-tocopherols, γ-tocopherols, δ-tocopherol and lutein from degradation during the sample treatment process. In other words, it was not necessary to protect the analytes from light

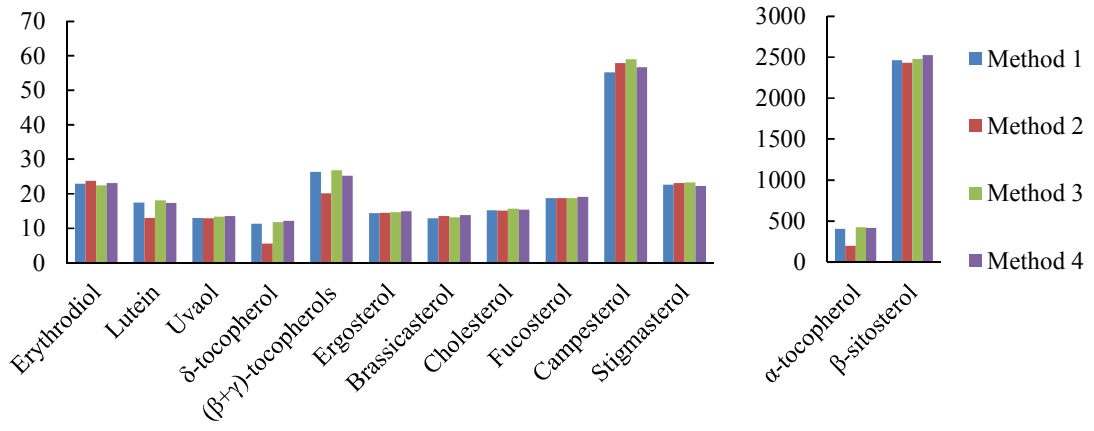

Fig. 3 Comparison of results of four different sample treatment methods. Method 1: Protect from light during sample treatment, and without using L-ascorbic acid sodium salt; Method 2: With light during sample treatment, and without using L-ascorbic acid sodium salt; Method 3: Protect from light during sample treatment, and with the use of L-ascorbic acid sodium salt; Method 4: With light during sample treatment, and with the use of L-ascorbic acid sodium salt.

Table 2 Linear range, linear regression equation, R^2, LOD and LOQ for the 14 analytes

Analytes	Linear range (mg L^{-1})	linear regression equation	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Erythrodiol	0.05 - 1.00	y=60458x-1395	0.9933	0.010	0.050
Lutein	0.05 - 1.00	y=100094x-168	0.9960	0.005	0.030
Uvaol	0.05 - 1.00	y=74568x-3297	0.9946	0.025	0.120
δ- tocopherol	0.05 - 1.00	y=5731x-107	0.9991	0.050	0.200
(β+γ)- tocopherols	0.05 - 1.00	y=13911x-92	0.9999	0.020	0.100
Ergosterol	0.05 - 1.00	y=55699x-305.2	0.9997	0.025	0.120
α-tocopherol	0.50 - 10.0	y=16640x+3228	0.9998	0.100	0.500
Brassicasterol	0.05 - 1.00	y=259026x-993.9	0.9998	0.003	0.010
Cholesterol	0.05 - 1.00	y=287046x+3032	0.9998	0.005	0.020
Fucosterol	0.05 - 1.00	y=619557x-70256	0.9999	0.003	0.020
Campesterol	0.125 - 2.50	y=516817x+5964	0.9994	0.003	0.010
Stigmasterol	0.05 - 1.00	y=407929x+11188	0.9977	0.025	0.100
β-sitosterol	5.00 - 100	y=360332x+39827	0.9999	0.050	0.200

during the sample treatment process when the L-ascorbic acid sodium solution was added into the olive oil samples before saponification. Therefore, the use of L-ascorbic acid sodium salt made the sample treatment became a facile procedure, in which we no longer need to use amber glass tubes or enwrap the tubes. Thus, the use of L-ascorbic acid sodium salt made the sample treatment became a facile procedure, we no longer need to carry out the laborious tube wrapping work.

The use of L-ascorbic acid sodium salt could protect the analytes, but would increase the complexity of matrix and cause instrument contamination or matrix effect. So the amount of L-ascorbic acid sodium solution that added into olive oil samples needed to be optimized. Five sets of spiked olive oil samples were added with 0.05 mL, 0.10 mL, 0.25 mL, 0.5 mL and 1.0 mL of L-ascorbic acid sodium solution (0.2 g mL^{-1}), respectively. Theses samples were treated with light and analyzed by LC-MS, and finally we found that the amount of 0.25 mL was the best. The content of α-tocopherol as well as δ-tocopherol decreased slightly when the amount of L-ascorbic acid sodium solution was less than 0.25 mL, while they kept constants when the amount was more than 0.25 mL.

3.4 Method validation

The parameters evaluated for the validation of the method were linearity of the standard curves, the detection and quantification limits, recovery, repeatability and reproducibility.

3.4.1 Linear range and detection limits

Table 3 Precision of the LC-MS method

Analytes	Intra-sample (n = 6)	Intra-day (n = 6)	Inter-day (n = 5)
	mg kg^{-1} (RSD%)		
Erythrodiol	22.54 (3.1)	22.97 (6.5)	23.82 (8.1)
Lutein	17.68 (2.8)	18.03 (5.1)	18.65 (7.6)
Uvaol	13.24 (4.3)	12.96 (7.6)	14.07 (10.1)
δ-tocopherol	12.67 (3.9)	12.34 (5.2)	12.88 (13.6)
(β+γ)-tocopherols	24.56 (4.5)	23.17 (8.2)	24.18 (11.7)
Ergosterol	14.25 (1.6)	14.69 (5.6)	14.87 (8.9)
α-tocopherol	409.6 (2.7)	418.4 (7.3)	422.3 (10.5)
Brassicasterol	14.26 (1.2)	15.98 (7.6)	15.16 (9.4)
Cholesterol	14.68 (1.9)	15.42 (4.8)	15.68 (5.9)
Fucosterol	18.94 (2.5)	18.24 (4.6)	19.50 (6.1)
Campesterol	54.23 (3.8)	52.43 (6.9)	55.87 (8.8)
Stigmasterol	23.28 (3.7)	23.84 (8.6)	24.76 (11.0)
β-sitosterol	2498 (2.3)	2546 (5.3)	2571 (9.3)

Calibration curves for every analytes were obtained by triplicate injections of multi-compounds working standard solutions at concentration levels ranging from 0.05 to 1.0 mg L^{-1} for erythrodiol, lutein, uvaol, γ-tocopherol, δ-tocopherol, stigmasterol, brassicasterol, cholesterol, ergosterol and fucosterol, from 0.125 to 2.5 mg L^{-1} for campesterol, from 0.5 to 10 mg L^{-1} for α-tocopherol and from 5.0 to 100 mg L^{-1} for β-sitosterol. β-tocopherol was not included in the multi-compounds working standard solutions, since it would be quantified together with γ-tocopherol. The linear ranges, linear regression equations and the corresponding correlation coefficients (R^2) were listed in Table 2. We can see that the calibration curves obtained for analytes showed good linearity in the range tested with R^2 higher than 0.994 in all cases.

Limits of detection (LOD) and limits of quantification (LOQ) were determined as the lowest concentration level that yielded a signal-to-noise (S/N) ratio of 3 and 10, respectively. These values ranged from 0.003 to 0.100 mg L^{-1} for LOD and 0.010 to 0.500 mg L^{-1} for LOQ, as listed in Table 2.

3.4.2 Precision

The precision of the LC-MS method of the combined extraction and LC-MS method were evaluated by performing intra-sample, intra-day and inter-day analysis of a spiked olive oil sample mentioned in section 3.3. The intra-sample analysis was performed by injecting the same extraction for six times with the same instrumental condition. Six samples of the same spiked olive oil were extracted and injected separately on the same day to determine the intra-day repeatability, while five samples were extracted and injected separately on five sequential days with different operators to determine the inter-day reproducibility.

As listed in Table 3, the relative standard deviations (RSD) of intra-sample injections (n = 6) were below 5.0%, indicating good precision of the LC-MS method. Due to the added variable of sample treatment, the intra-day (n = 6) and inter-day (n = 5) analysis had slightly higher RSD values of ≤8.6% and ≤13.6%, respectively, remaining acceptable below the targeted <15%.

Table 4 Recoveries of analytes from spiked samples (n = 3)

Analyte	Blank sample (mg kg^{-1})	Spiking amount (mg kg^{-1})	Recovery (%)	RSD (%)
Erythrodiol	8.369	4	75.62	5.6
		8	85.91	4.7
		16	86.04	6.1
Lutein	3.263	4	81.38	3.5
		8	84.85	4.2
		16	92.19	1.8
Uvaol	ND	4	78.60	7.2
		8	84.53	6.6
		16	80.11	4.3
δ- tocopherol	ND	4	95.35	4.8
		8	85.97	3.5
		16	82.66	4.4
(β+γ)-tocopherols	13.04	4	88.32	7.0
		8	84.91	3.6
		16	85.58	4.8
Ergosterol	1.578	4	84.47	4.1
		8	91.79	6.1
		16	83.58	5.7
α-tocopherol	302.5	40	105.8	3.7
		80	89.53	7.0
		160	90.44	4.6
Brassicasterol	ND	4	82.87	6.0
		8	91.92	3.9
		16	87.31	5.6
Cholesterol	ND	4	93.44	6.9
		8	96.02	3.0
		16	92.51	4.6
Fucosterol	6.524	4	86.88	4.5
		8	91.67	6.8
		16	85.22	5.2
Campesterol	27.29	10	94.63	7.8
		20	89.11	6.2
		40	89.70	4.4
Stigmasterol	7.412	4	85.48	7.6
		8	88.87	5.1
		16	92.50	5.3
β-sitosterol	876.0	400	94.51	4.7
		800	90.76	4.2
		1600	103.2	3.9

ND: not detected.

3.4.3 Recovery

Table 5 The results of comparison of current method with existing methods

Analytes	Olive oil sample 1		Olive oil sample 2		Olive oil sample 3		Olive oil sample 4		Olive oil sample 5	
	Proposed method	Other methods	Proposed method	Other methods	Proposed method	Other methods	Proposed method	Other methods	Proposed method	Other methods
	mg kg^{-1}									
Erythrodiol	8.369	8.886[a]	17.09	16.50[a]	5.485	5.893[a]	10.25	9.551[a]	16.24	15.86[a]
Lutein	3.263	3.024[c]	3.172	2.979[c]	2.568	2.332[c]	3.649	3.458[c]	2.854	2.638[c]
Uvaol	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]
δ- tocopherol	ND	ND[b]	ND	ND[b]	ND	ND[b]	ND	ND[b]	2.866	3.310[b]
(β+γ)-tocopherols	13.04	14.76[b]	6.576	7.392[b]	9.643	10.62[b]	15.93	17.65[b]	12.53	13.25[b]
Ergosterol	1.578	1.552[a]	1.609	1.648[a]	1.867	1.724[a]	1.245	1.336[a]	2.569	2.663[a]
α-tocopherol	302.5	336.4[b]	289.2	328.8[b]	268.1	287.5[b]	326.0	359.4[b]	246.1	280.6[b]
Brassicasterol	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]
Cholesterol	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]	ND	ND[a]
Fucosterol	6.724	7.120[a]	7.671	8.300[a]	8.164	7.753[a]	6.827	7.263[a]	7.260	6.823[a]
Campesterol	27.29	29.60[a]	31.18	34.20[a]	25.92	25.45[a]	33.61	36.27[a]	30.56	32.59[a]
Stigmasterol	7.412	7.120[a]	6.934	6.360[a]	6.593	7.124[a]	6.225	6.824[a]	7.694	7.867[a]
β-sitosterol	876.0	952.4[a]	1089	1148[a]	1068	1158[a]	994.3	983.2[a]	1126	1226[a]

ND: not detected.

a: detected via the gas chromatographic method of International Olive Oil Council COI/ T.20/ Doc. No 30-2013.

b: detected via the normal phase HPLC method of ISO 9936:2006.

c: detected via the HPLC method described by Darnoko et al.[22].

The recovery of the extraction method was determined by the use of three sets of spiked olive oil samples (three spiking levels, each analyzed in triplicate). The spiking amounts and the recoveries of analytes were listed in Table 4. The recoveries of all 14 analytes ranged from 75.62% to 105.8%, indicating good recovery of the method. In the reported work, tocopherols were found to have lower averaging recoveries due to the degradation or oxidation during sample treatment[12]. Benefitting from the use of L-ascorbic acid sodium salt in sample treatment process, tocopherol recoveries (>80%) were as high as other analytes in our work.

3.5 Comparison of current method with existing methods by analyzing real samples

To validate the consistency of the proposed method to other methods, five extra virgin olive oil samples were analyzed via the current methods and the previous published methods. Phytosterols, erythrodiol and uvaol were detected via the gas chromatographic method of International Olive Oil Council COI/T.20/Doc. No 30-2013. Tocopherols were detected via the normal phase HPLC method of ISO 9936:2006. For lutein, the HPLC method described by Darnoko et al.[22] was used. The results of the various methods were presented in Table 5. The results from these methods were in strong consistency for phytosterols, erythrodiol, uvaol and lutein, all of which varied by less than 10% between different methods and showed similar trends in values among samples. Tocopherols showed slightly higher variations compared with the official method, but still in a reasonable range (<15%). These results demonstrated that the present method was accurate for analyzing phytosterols, erythrodiol, uvaol, tocopherols and lutein in olive oils.

4 Conclusions

In conclusion, this work offers a facile method for simultaneous analysis of four classes of unsaponifiable fraction in olive oils – phytosterols, triterpene dialcohols (erythrodiol and uvaol), tocopherols and lutein. The separation of erythrodiol and uvaol was achieved for the first time in HPLC experiments, and thus the two analytes were quantified separately. β- and γ-tocopherols were quantified together because they had the same retention time and provided the same *m/z* in MS. The use of L-ascorbic acid sodium salt made the sample treatment became a facile procedure, in which we no longer need to protect the analytes from light. Besides, L-ascorbic acid sodium salt is more environmental friendly as an antioxidant compared with TBHQ. The amount of solvents we used in sample treatment were much less than those in standard methods such as International Olive Oil Council COI/T.20/Doc. No 30-2013. Therefore, we proposed a facile, environmental friendly and sensitive method to analyze more analytes in a single procedure.

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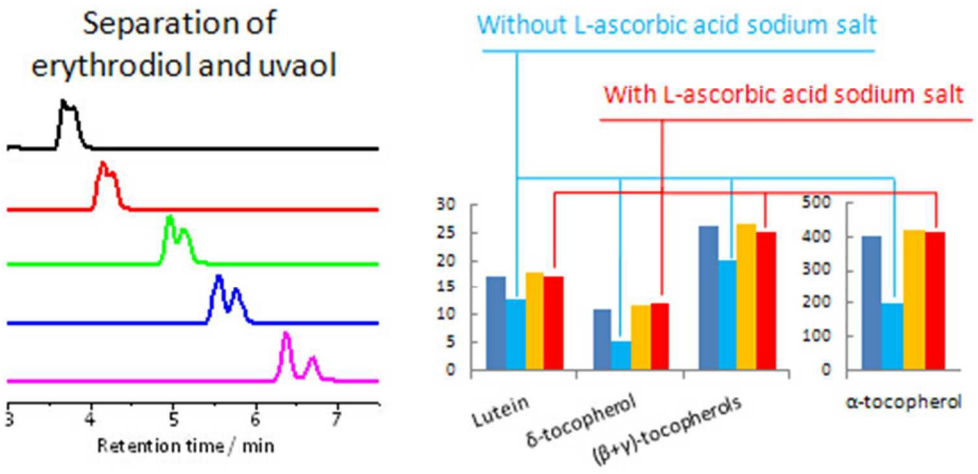

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