Scientific Papers of the University of Pardubice,
Series A; Faculty of Chemical Technology
26 (2020) 111–124.

UNIVERSITY OF PARDUBICE
FACULTY OF CHEMICAL TECHNOLOGY

Determination of oleocanthal in extra virgin olive oils using high-performance liquid chromatography

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Received: June 2, 2020; Accepted: June 25, 2020

This work is focused on the determination of oleocanthal in nineteen extra virgin olive oils plus one sample of the olive pomace oil using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled either with a spectrophotometric detector (set at λ = 275 nm) or a mass spectrometer. Sample pre-treatment including a two-step liquid-liquid extraction by polar solvents was performed and thoroughly optimised. The extract of oleocanthal was analysed using Poroshell 120 EC-C18 column (150 × 3 mm, i.d. 2.7 µm) in combination with the binary mobile phases consisting of water and acetonitrile. The sum of oleocanthal hemiacetals was quantified by the calibration curve method and the standard addition method. The content of oleocanthal in the samples was found within a range of 2.8–27.2 mg/100 g. It is shown that the method can also be used for evaluation of the olive oil quality.

Keywords: Oleocanthal; Extra virgin olive oil; Extraction; Liquid chromatography.

Introduction

Extra virgin olive oil (EVOO) is a sort of vegetable oil made from the fruit of the plant *Olea Europaea* [1]. For the EVOO preparation, the prescribed technological procedure must be strictly followed. Only high-quality, fresh, and intact olives of precise ripeness are collected, from which the oil is obtained exclusively by cold pressing, even without prior crushing of the olives. The use of any additives is strictly forbidden throughout the EVOO production process. Furthermore, only defined mechanical processes, including decantation, centrifugation, and filtration, are allowed to treat the pressed EVOO [2–6].

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The composition of EVOO is affected by many factors that determine its final quality and sensory, nutritional, physical, and chemical properties. The olive cultivar, pedoclimatic conditions of its growth (the presence of natural moisture, sun intensity, the altitude and latitude plus the quality and composition of soil), technological aspects of olive growing (fertilization and irrigation), as well as the ripeness and processing of cultivated olives, are the most important parameters and factors influencing the EVOO composition [7–10].

EVOO is a source of a wide range of substances, among which di- and tri-acylglycerols (99 %), and free fatty acids (FA) are the most abundant items [11]. The minor substances then include up to 230 chemical compounds belonging to aliphatic and di- and tri-terpenic alcohols, vitamins, volatile compounds, sterols, pigments, and other biologically active substances, such as phenolic compounds [5,12–17]. Although, only 2 % of polyphenols are transferred from the olives into the oil during the process, extraction of EVOO by polar solvents gives rise to a phenolic fraction containing nearly 40 structurally different phenolic compounds, such as phenolic acids and alcohols, secoiridoids, and flavonoids [7,16,18–23].

Oleocanthal (OCA) is a secoiridoid composed of tyrosol and 4-decarboxy methyl elenolic acid. This substance is presented exclusively in EVOO and has many benefits on human health, among which one can quote antimicrobial, anti-inflammatory, analgesic, or anti-cancer properties, positive effects on neuro-logical degenerations, atherosclerosis, rheumatoid arthritis, and cardiovascular diseases. Moreover, OCA has also been shown to protect the body against Alzheimer's disease [24–27] and it is reported that OCA provides a similar medical effect as *Ibuprofen*® [28,29]. On the other side, OCA is known for its high instability in the presence of the light and in contact with some polar solvents and also, it is readily decomposed by alcohols forming the respective hemiacetals [26,29].

Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a spectrophotometric (UV/Vis) detection is the most applied technique for the OCA extract analysis. The separation is performed using non-polar C18-columns combined with the binary mobile phases usually containing mixtures of water with methanol or acetonitrile [7,23,25,26,29,30].

The aim of this study was to develop an analytical method for the oleocanthal determination in EVOOs comprising the inevitable extraction step, chromatographic separation, and UV/Vis detection.

Experimental

Chemicals and Reagents

Standard of OCA (≥ 98 %) was purchased from PhytoLab (Vestenbergsgreuth, Germany). Solvents, such as methanol and acetonitrile (both LC/MS purity) used for extraction and preparation of mobile phases, and *n*-hexane (≥ 95 %) as

extracting medium, were purchased from Sigma-Aldrich (Prague, Czech Republic). Demineralised water was prepared in a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

Twenty one oils analysed are listed in Table 1. Most of the samples were purchased in common Czech stores (Kaufland, Billa, Globus, Lidl, and Tesco) and classified as the EVOOs. One of the samples (No. 20) was made from olives' pomace and originated from Spain. Another one (No. 21) obtained by cold pressing of rape seeds was purchased from a local producer (Bio Energo Komplex, Kolín, Czech Republic). Finally, the rapeseed oil was used as the reference non-OCA standard.

Table 1 Specification of a set of twenty one oils selected for analysis

Sample No.	Name	Type of oil	Country of origin
1	Evlogimeno	EVOO	Greece
2	La Española	EVOO	Spain
3	Borges	EVOO	E.U. mixture
4	Italiamo	EVOO	Italy
5	Monini	EVOO	Italy
6	Kaiser Franz Josef	EVOO	E.U. mixture
7	Terra Delyssa	EVOO	Tunisia
8	Ondoliva	EVOO	Spain
9	Colavita	EVOO	Italy
10	Iliada Kalamata	EVOO	Greece
11	Demetra	EVOO	Greece
12	Caballero	EVOO	E.U. mixture
13	Seville Preimum	EVOO	Spain
14	Ballester	EVOO	Spain
15	Filippo Berio	EVOO	E.U. mixture
16	Terra Creta	EVOO	Greece
17	Alnatura	EVOO	Tunisia
18	K-bio	EVOO	Spain
19	Costa d´Oro	EVOO	Italy
20	Giana	Pomace olive oil	Spain
21	Rapeseed oil	Rapeseed oil	Czech Republic

The EVOOs analysed had a different origin: four were a blend of olive oils from unspecified countries within the European Union (samples No. 3, 6, 12, 15), four EVOOs came from Greece (sample No. 1, 10, 11, and 16), four from Italy (sample No. 4, 5, 9, 19), two EVOO samples were from Tunisia (sample No. 7

and 17), and the remaining five from Spain (samples No. 2, 8, 13, 14, 18). However, the available information did not specify whether the oils came from one producer in the respective country or they were a mixture collected at several locations.

All the oil samples were stored in a refrigerator and tempered at the room temperature before analysis.

Instrumentation

The HPLC system equipped with a LC-20ADXR binary gradient pump, a DGU-20A degassing unit (all Shimadzu, Kyoto, Japan) and a six-port valve with 2 μL or 10 μL external loop (Valco-Vici, Schenkon, Switzerland) was coupled with a SPD-20A spectrophotometric detector (Shimadzu) for quantitative analysis of OCA in EVOOs. Poroshell 120 EC-C18 column (150 × 3 mm, i.d. 2.7 μm; Agilent, Santa Clara, CA, USA) was situated in a LCO 102 column thermostat (Ecom, Prague, Czech Republic). The HPLC-MS system equipped with the same brands of pumps, column, thermostat and degassing unit but coupled with a SIL-20A autosampler (Shimadzu) and a QTRAP 4500 mass spectrometer (AB Sciex, Framingham, MA, USA) was used for qualitative analysis and confirmation of the OCA hemiacetals in the samples. Measurements in the ultraviolet region were carried out with a UV-2600 spectrophotometer (Shimadzu) using 1 cm quartz cuvette (Fisher Scientific, Pardubice, Czech Republic) in the wavelength range from 200 to 400 nm.

Preparation of standard solution

A standard solution of OCA with the concentration of 0.5 mg mL^{-1} in acetonitrile was prepared and stored in a refrigerator.

Extraction procedure

OCA was extracted from EVOO using slightly modified method previously described by Impellizzeri and Lin [29]. An amount of 5 g EVOO was mixed with 10 mL of *n*-hexane in a centrifuge tube and extracted with 25 mL of 100% acetonitrile. After 15 min of stirring (REAX 2 shaker, Heidolph, Germany), the emulsion was centrifuged at 4000 rpm for 5 min (Universal 320 centrifuge, Hettich, Germany). The polar organic phase with extracted phenolic compounds was separated, evaporated at 35 °C (Digital Dry Bath, Miulab, Hangzhou, China) by the stream of nitrogen, re-dissolved in 10 mL of *n*-hexane, and re-extracted by 5 mL of 50 % methanol in water (v/v). The extract obtained in this way was stirred

again for 15 min and centrifuged at 4000 rpm for 5 min. The polar water-organic layer was separated, filtered through a PTFE syringe filter (0.22 µm; Labicom, Olomouc, Czech Republic), and analysed.

HPLC analysis

Separation conditions were optimised for the HPLC analysis of the extracted OCA hemiacetals. Final separation was performed on the Poroshell 120 EC-C18 column with gradient elution using binary mobile phase consisting of water (A) and 100 % acetonitrile (B). The gradient program was following: 0 min – 27 % B, 10 min – 50 % B, 11 min – 27 % B. The flow rate was 0.5 mL min^{-1}, temperature 40 °C and injection volume 10 µL. Absorption wavelength was set to $\lambda = 275$ nm. The optimised separation conditions were used also for HPLC-MS analysis, only the injected volume was lower (2 µL) in this case. HPLC-MS experiments were performed in both positive and negative ion mode under the following operating conditions: electrospray ionization; curtain gas: 20 psi, collision gas: medium, ion spray voltage: ± 4500 V, temperature: 450 °C, first ion source gas: 40 psi, second ion source gas: 50 psi, entrance potential: ±10 V, declasteration potential: ±90 V. Clarity software (DataApex, Prague, Czech Republic) was used for data collection and evaluation.

Oleocanthal quantification

The calibration curve method and standard addition method were used for the determination of OCA in oil samples. The volume ranging from 200 to 2800 µL OCA standard solution with the concentration of 0.5 mg mL^{-1} was added to 5 g of OCA free rapeseed oil matrix. These fortified oil samples were extracted and analysed by optimised method thoroughly as described above. The peak areas of two OCA hemiacetal forms were summarised and calibration curve constructed (Fig. 1). Each calibration concentration level together with oil samples were processed in duplicate and each extract obtained was analysed three times by HPLC-UV/Vis ($n = 6$). Calibration regression equation of OCA with values of its standard deviations are specified by inset in Fig. 1.

A similar procedure was used for the OCA quantification in selected samples by the standard addition method. At first, an EVOO sample without any fortification step was processed and analysed, and peak area of OCA recorded. Then, the OCA standard solution with the volume 0.3 mL ($c = 0.5$ mg mL^{-1}) was added to the EVOO samples before their extraction. After processing and analysis, the OCA concentration was calculated from the increasing peak area. The standard addition was performed in duplicate and each extract was measured three times.

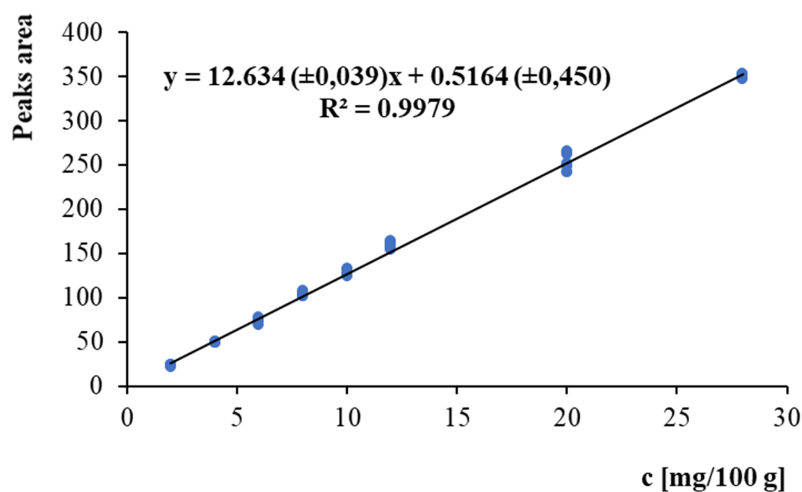

Fig. 1 Calibration curve of oleocanthal with the corresponding regression equation and related data

Results and discussion

Spectrophotometric analysis of oleocanthal isomers

OCA is known for the formation of hemiacetal forms in the presence of some polar solvents, especially alcohols [26,29]. The scheme of the hemiacetal formation is displayed in the Fig. 2. Therefore, the influence of OCA solvent (50% acetonitrile in water or 50% methanol in water) on UV/Vis spectrum was investigated. Nevertheless, both spectra showed a similar profile with absorption maximum at 220 nm and 275 nm (Fig. 3); thus, the absorption spectrum is not affected by hemiacetals formation.

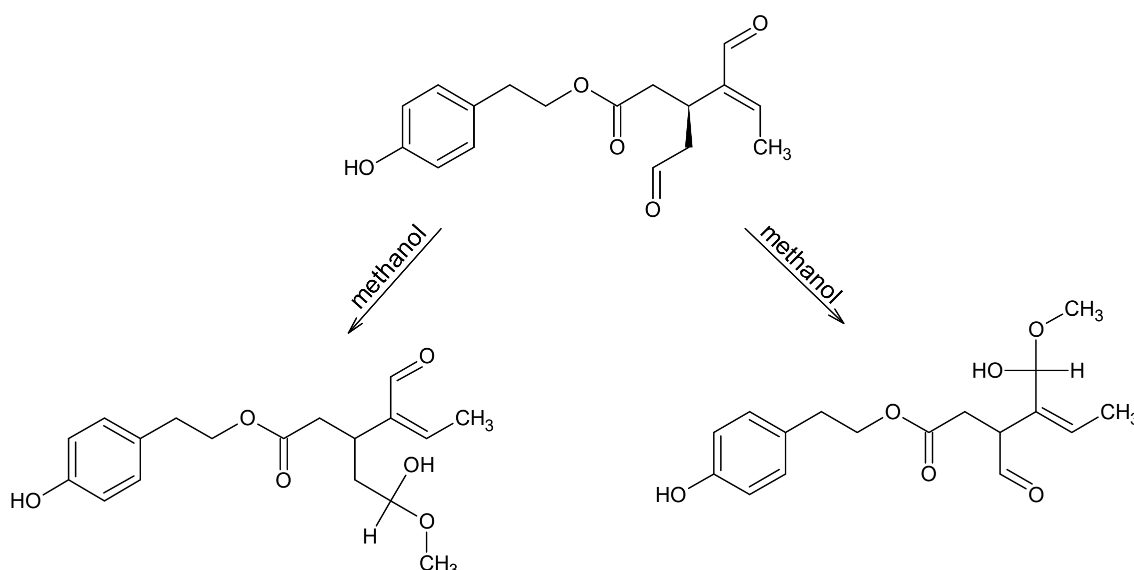

Fig. 2 Scheme of oleocanthal hemiacetals formation

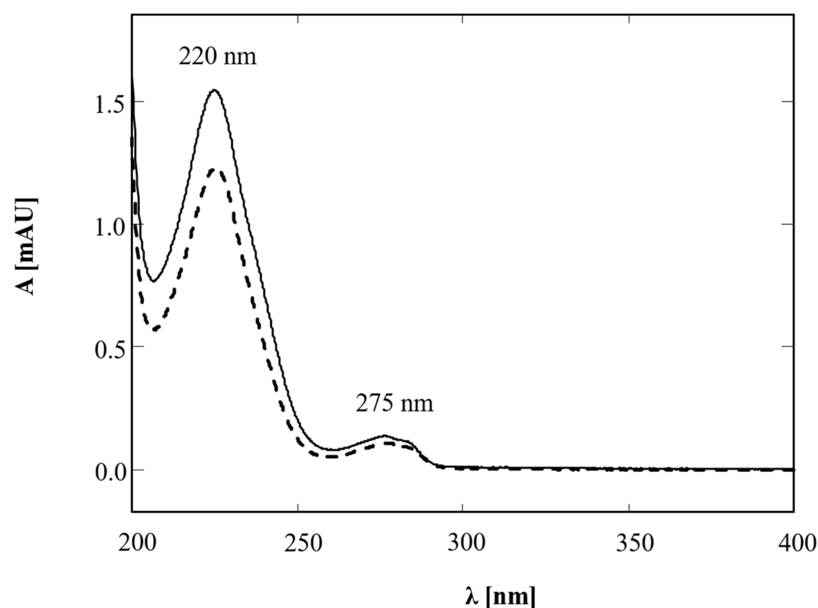

Fig. 3 UV spectrum of oleocanthal in 50% methanol in water (normal line) and in 50% acetonitrile in water (dashed line)

Although, the absorption maximum at 275 nm is not as intensive as that one at 220 nm, this wavelength was used for the detection of OCA by HPLC-UV/Vis instrumentation due to certain minor interferences revealed in the analysis of real samples.

Optimisation of the extraction procedure

Several methods of OCA extraction from olive oils have already been reported in the literature [26,27,29,30]; the approach reported in [29] having employed a two-step extraction step. The first step had involved dissolution of oil in hexane followed by the extraction of OCA onto pure methanol or acetonitrile. After stirring and centrifugation, the obtained extract was evaporated to dryness, the residue re-dissolved in *n*-hexane, and finally re-extracted with 100% acetonitrile or with 50% methanol in water.

As previously described by some authors [29], the choice of extraction reagent significantly affects the profile of the chromatogram obtained. Hence, two extraction methods were evaluated for optimisation of OCA isolation. The amount of 5 g rapeseed oil was fortified with 300 μL of OCA standard (c = 0.5 mg mL^{-1}) and first extraction step performed by acetonitrile as described in *Experimental*. In the second extraction step, either the 100% acetonitrile or 50% methanol in water was used (Fig. 4). From this figure, it is evident, that small deformed peak of OCA at retention time 6.2 min appears when acetonitrile is

used. On the other hand, the mixture of methanol and water suppresses the OCA peak in behalf of its hemiacetals (a pair of peaks with retention around 4.3 min). Due to better detector responses of OCA hemiacetals, 50 % methanol was used in the second step for all the remaining experiments.

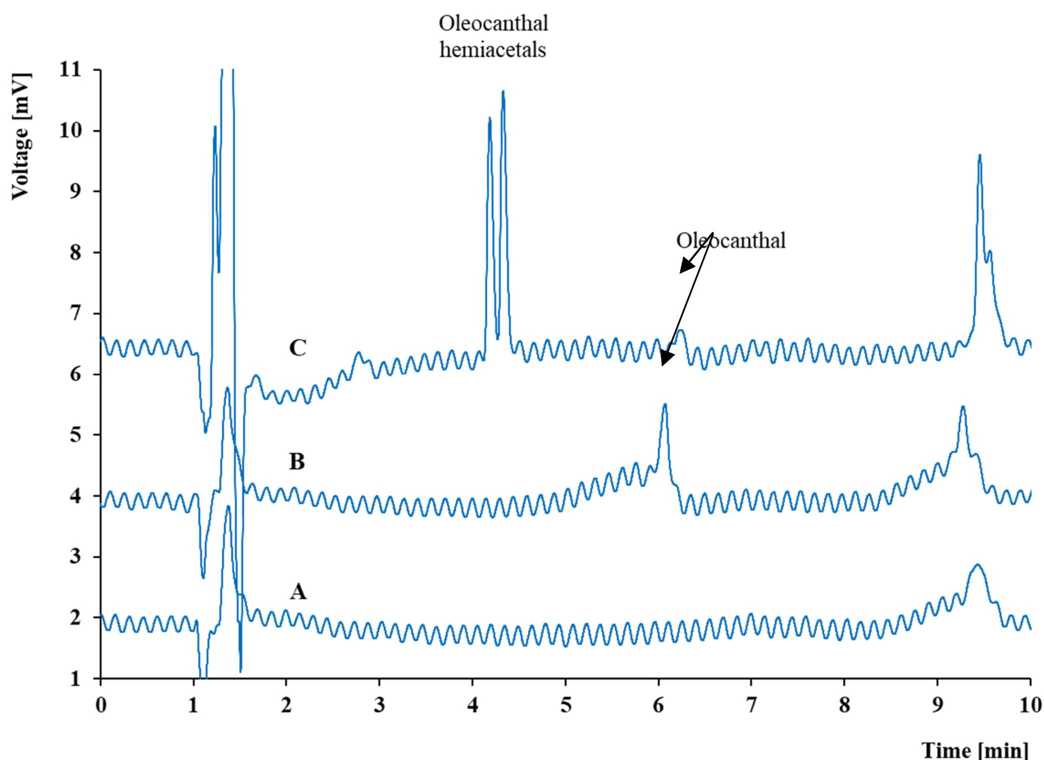

Fig. 4 Extract of not fortified (A) and fortified (B) rapeseed oil using 100% acetonitrile in both extraction steps, and extract of fortified rapeseed oil using 100% acetonitrile in the first step and 50% methanol in water (v/v) in the second step (C)

Separation conditions: Poroshell 120 EC-C18 (150 × 3 mm, i.d. 2.7 μm); 40 °C; 0.5 mL min^{-1}; gradient elution 27–50 % of acetonitrile in water in 10 min; λ = 275 nm.

Optimisation of HPLC analysis

Parameters such as type of an organic component of the mobile phase (acetonitrile or methanol), as well as various gradient slope with different initial and final concentration were optimised. It was found that the best separation with symmetric and narrow peaks was obtained with binary mobile phase comprised of water (A) and 100 % acetonitrile (B) with following gradient elution program: 0 min – 27 % B, 10 min – 50 % B, 11 min – 27 % B. HPLC chromatogram of EVOO sample No. 4 is depicted in the Fig. 5.

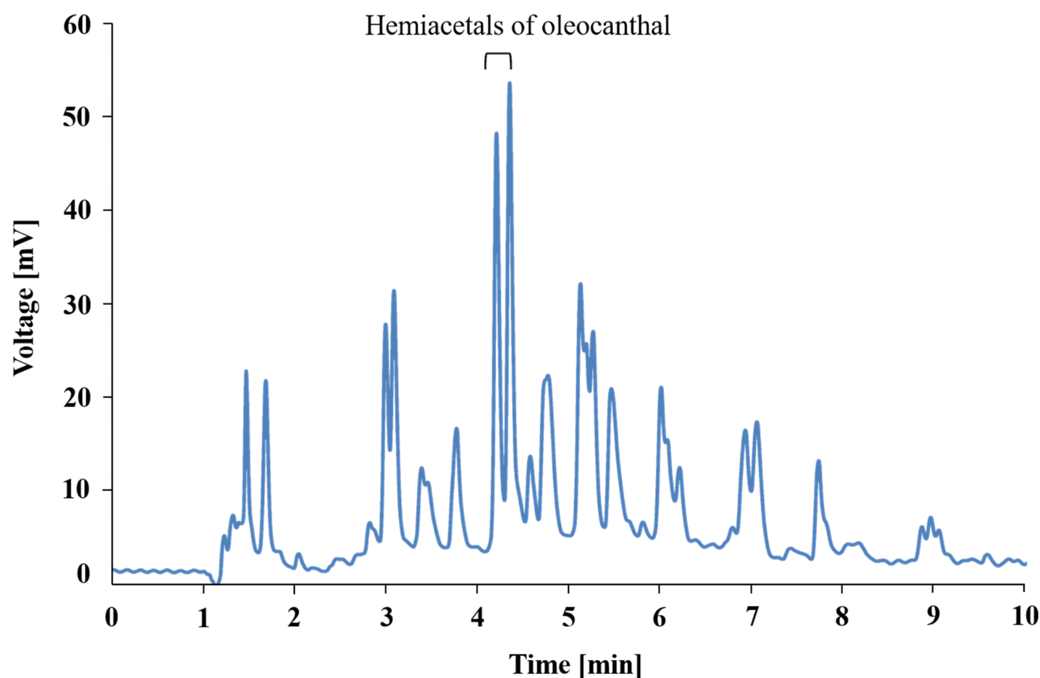

Fig. 5 Chromatogram of the EVOO sample No. 4

Separation conditions: Poroshell 120 EC-C18 (150 × 3 mm, i.d. 2.7 μm); 40 °C; 0.5 mL min^{-1}; gradient elution 27–50 % of acetonitrile in water in 10 min; λ = 275 nm.

From Fig. 5, it is evident that many other polar substances, especially polyphenols, are co-extracted with OCA during its isolation by the liquid-liquid extraction. Therefore, HPLC-MS analysis of the pomace olive oil extract, as well as analysis of extract of rapeseed oil fortified by OCA, was performed for confirmation of OCA hemiacetals. These substances with the molecular weight M = 336 g mol^{-1} were found in both positive and negative ion mode and both hemiacetal forms provided completely the same mass spectra. In the negative-ion mode, the following ions were observed: m/z = 303, $[M–CH_3OH–H]^-$; m/z = 671, $[2M–H]^-$; m/z = 335, $[M–H]^-$; m/z = 183, $[M–CH_3OH–C_8H_8O–H]^-$; m/z = 233, $[M–CH_3–CH=CH–CHO–H]^-$; m/z = 285, $[M–CH_3OH–H_2O–H]^-$; and m/z = 259, $[M–CH_3OH–CO_2–H]^-$. In the positive-ion mode, we could then observe: m/z = 359; $[M+Na]^+$; m/z = 375, $[M+K]^+$; m/z = 695, $[2M+Na]^+$; m/z = 319, $[M–H_2O+H]^+$; m/z = 305, $[M–CH_3OH+H]^+$; and m/z = 120, $[C_6H_4(OH)CH_2CH_2]^{+\bullet}$.

Sample analysis and data validation

The calibration curve method was used for determination of the OCA content in all the EVOO samples.

First, the rapeseed oil was analysed to confirm the absence of OCA. Then, 100 g of rapeseed oil was fortified with OCA in a concentration range 2–28 mg/100 g, for which a calibration series was prepared. Each concentration level of fortified rapeseed oil underwent the entire process of the sample pre-treatment described in the *Experimental*. Thus, a more accurate quantification of OCA could be determined by including the inaccuracy associated with random errors occurring during the extraction. The calibration dependence plotted and characterized by regression data was used to determine the OCA content in the olive oil samples (see again Fig. 1 and inset in); the relative standard deviation (±0.45) of intercept (0.5164) reveals its statistical insignificance.

Further, the method for the OCA determination was validated in the terms of linearity and limits of detection (LoD) and quantification (LoQ), respectively. The linearity of the calibration curve expressed as a coefficient of determination (R^2) was sufficient and represented a value of $R^2 = 0.9979$. LoD and LoQ were determined by estimating the concentration according to the signal-to-noise ratio (S/N) of 3 or of 10; the calculated values of LoD and LoQ being 0.57 mg and 1.9 mg of OCA in 100 g of oil, respectively.

The results of analyses of all the samples are summarized in Fig 6. From the data surveyed, no clear conclusions can be drawn about the relation between the OCA content and the country of the oil production. Nevertheless, it can be seen that the concentration of OCA is more affected by botanical (olive cultivar) and geographical (latitude and longitude) origin of the olives, by their degree of ripeness, agricultural practices (watering, fertilization, soil type, and quality), the climate, as well as the whole production process including final storage of the oil [16,18,24,25]. Thus, country of origin does not play a crucial role. In all analysed samples of EVOO, the concentration of OCA was found in an interval from 2.8 to 27.2 mg/100 g, with the average value of 8.8 mg/100 g. The absolutely highest OCA content was observed in Italian EVOO No. 4 of the *Italiamo* brand purchased in Lidl market (27.2 mg/100 g).

As illustrated in Fig. 6, the OCA content was below LoD in the pomace oil (sample No. 20). This oil is produced by extraction of the olives´ pomace after its pressing. Since the extraction is performed using nonpolar solvents like aliphatic hydrocarbons, most of the phenolic compounds are not transferred to the pomace oil. For this reason, the absence of OCA is expected.

For the selected oils (No. 1, 6, and 8), the concentration of OCA was also determined using the standard addition method, the results obtained by both methods were compared and given in the Table 2.

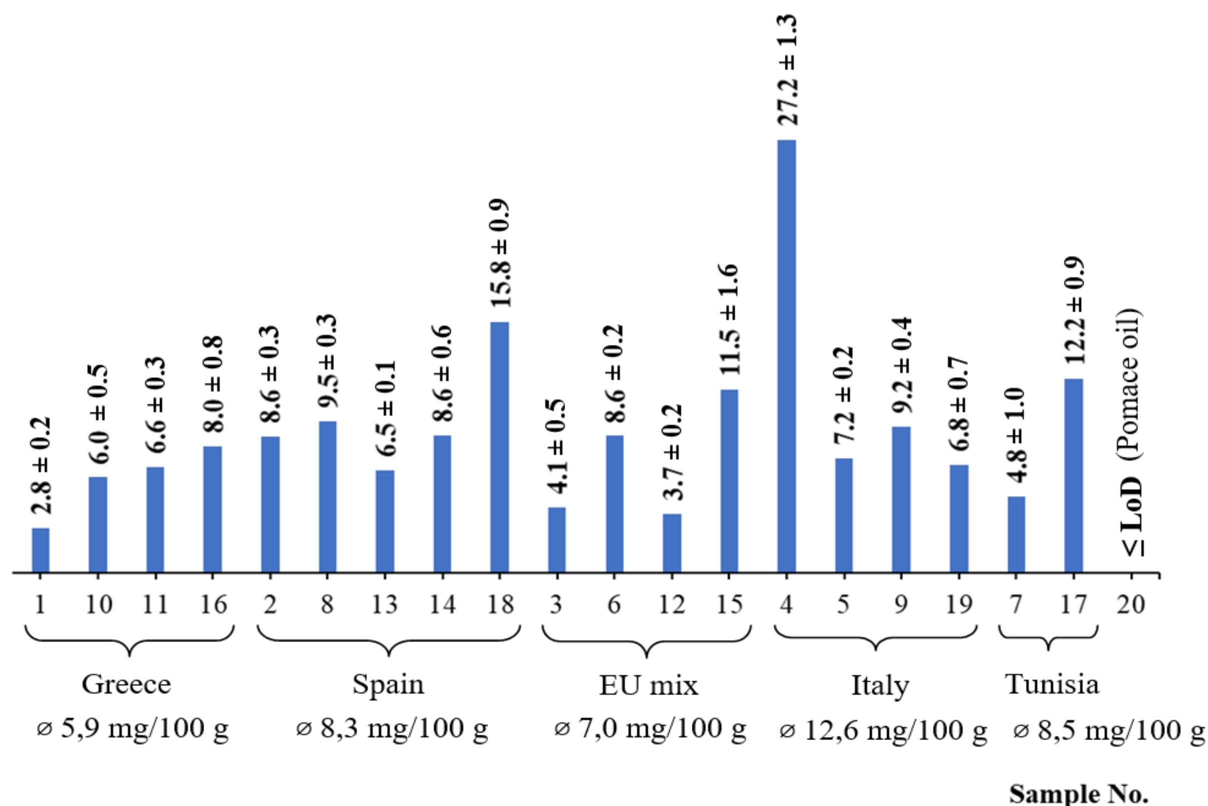

Fig. 6 Bar chart of determined amount of oleocanthal [mg/100 g] in 19 samples of EVOO (No. 1–19) and one sample of oil from olive pomace (No. 20)

Table 2 Comparison of oleocanthal concentration [mg/100 g] in 3 EVOOs determined by calibration curve method (CCM) and method of standard addition (SAM)

	Sample No. 1		**Sample No. 6**		**Sample No. 8**	
Repetitions	CCM	SAM	CCM	SAM	CCM	SAM
1	3.0	3.3	8.8	10.2	9.4	7.9
2	3.1	2.9	8.8	9.1	9.2	7.3
3	2.9	2.7	8.6	8.3	9.0	8.3
4	2.7	2.4	8.5	8.1	9.7	10.8
5	2.8	3.0	8.4	7.3	9.8	10.4
6	2.5	2.7	8.5	7.1	9.9	11.3
Average with standard deviation	2.8 ± 0.2	2.8 ± 0.2	8.6 ± 0.1	8.4 ± 0.9	9.5 ± 0.3	9.3 ± 1.5

It has been found that there is no significant difference between the results acquired by the calibration curve method and those by using standard addition method. Nevertheless, the calibration curve method was found to lead to better values of standard deviations.

Conclusions

In this work, the method for determination of oleocanthal was developed, optimised, and applied to the 19 samples of extra virgin olive oil plus one olive pomace oil; all being purchased in local markets. Oleocanthal was analysed by high-performance liquid chromatography coupled with spectrophotometric detector after the previous two-step liquid-liquid extraction by acetonitrile in the first step and methanol/water (1:1; v/v) in the second step. Then, the oleocanthal content was determined and calculated by calibration curve method using oleocanthal-enriched rapeseed oil (naturally oleocanthal free), as well as by the standard addition method. In all analysed EVOO samples, the ascertained content of oleocanthal varied in a range of 2.8–27.2 mg/100 g; the highest value being found in Italian EVOO (sample No. 4, *Italiamo* brand, bought in Lidl). On the other hand, oleocanthal was not found in the olive pomace sample.

Analysis of twenty different samples of olive oil has also confirmed that the content of oleocanthal can be used as an indicator characterising the EVOO as such, when the products with higher concentration of oleocanthal can be considered to be associated with better quality.

Acknowledgement

The support received from the Faculty of Chemical Technology, University of Pardubice (project No. SGS-2019-003) is gratefully acknowledged.

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