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A Simple High-Performance Liquid Chromatography Method for the Determination of Throat-Burning Oleocanthal with Probated Antiinflammatory Activity in Extra Virgin Olive Oils

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A high-performance liquid chromatography (HPLC) method was developed to quantitatively analyze oleocanthal in extra virgin olive oils. Oleocanthal, a deacetoxy ligstroside aglycone, is known to be responsible for the back of the throat irritation of olive oils and to have probated antiinflamatory activity. Oleocanthal was isolated from small amounts of olive oil sample (1 g) by liquid−liquid extraction. Hexane−acetonitrile was found to be the best solvent system to extract oleocanthal from the oil matrix. The solvent extract was analyzed by reversed-phase HPLC with UV detection at 278 nm. Chromatogaphic separation of oleocanthal from other extracted compounds and of the two geometric isomers of oleocanthal was achieved by an elution gradient with acetonitrile and water. Both the external standard calibration curve and the internal standard calibration curve were established, and quantitation using both calibration curves gave essentially the same result. The reproducibility (RSD = 4.7%), recovery (>95%), and limit of quantitation (<1 μg/g) were also determined. Concentrations of oleacanthal in 10 selected throat-burning extra virgin olive oils were determined using the method (ranged from 22 to 190 μg/g) with external standard calibration.

KEYWORDS: Oleocanthal; antiinflammatory; throat irritation; high performance liquid chromatorgraphy

INTRODUCTION

High-quality extra virgin olive oils of certain European regions, especially parts of Italy, Spain, and Greece, are notorious for their desirable "peppery" note. This taste is described as a peppery bite in the back of the throat, which can force a cough, or as a rough, burning, or biting sensation in the throat. The nonsteroidal antiinflammatory drug ibuprofen elicits a similar unique throat irritation that is associated with coughing and throat catch, provoking the hypothesis that an irritant in olive oil might have some of the same pharmacological properties as ibuprofen (*1*). In collaboration with colleagues at the Monell Chemical Senses Center, it was recently found that oleocanthal in olive oil was responsible for the throat irritation (unpublished results) and that it is a potent nonsteroidal antiinflammatory agent, similar to that of ibuprofen (*1*). Oleocanthal was identified for the first time in olive oil by Montedoro et al. (*2*) through spectroscopic characterization, including NMR, IR, and UV data. Andrewes et al. (*3*) independently reported that the deacetoxy-ligstroside aglycone was responsible for the pungency associated with some extra virgin olive oils.

During our collaboration, it was necessary to determine the levels of oleocanthal in olive oil to correlate them with the throat-burning intensities. A simple high-performance liquid chromatography (HPLC) method was thus developed to determine the concentrations of oleocanthal in olive oil. Furthermore, observation of the antiinflammatory activity of oleocanthal raised the possibility that long-term consumption of oleocanthal might help to protect against some diseases due to its ibuprofen-like cyclooxygenase-inhibiting activity (*1*). Further studies to substantiate this hypothesis would be required, and a method for quantitative determination of oleocanthal in olive oil would be useful. Therefore, we report here the HPLC method for determination of oleocanthal in olive oil.

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MATERIALS AND METHODS

Oil Samples. Ten extra virgin olive oils of different degrees of throat-burning intensity were received in amber glass bottles from the Monell Chemical Senses Center and kept at room temperature until analysis. These extra virgin olive oils were purchased from local stores, and they were selected for analysis after a rough evaluation of their throat-burning intensities (methodology to be published elsewhere). Bertolli pure olive oil was purchased from a local supermarket and was opened for more than 1 year. Corn oil was freshly purchased from a local supermarket.

Standard Solutions. Oleocanthal standard was provided by Prof. Smith's research laboratory, where the compound was synthesized (*4*). Pure oleocanthal (1 mg) received was made into a solution of known concentration (1000 ppm) in toluene (1 mL) and kept frozen. Upon usage, an aliquot of the toluene solution was taken and the solvent was gently removed by a stream of N_2. Methanol/water (1/1, v/v) was added to make a stock solution, which can be used to spike a corn oil sample or to prepare calibration solutions. The internal standard (ISTD) 3,5-dimethoxyphenol was purchased from Aldrich. A stock solution (6790 ppm) of it was prepared in methanol, and a 200 ppm working

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Table 1. HPLC Mobile Phase Gradients

gradient 1			gradient 2		
time (min)	MeOH (%)	H_2O (2% HOAc) (%)	time (min)	ACN (%)	H_2O (%)
0	35	65	0	25	75
50	35	65	35	25	75
50.01	60	40	35.01	80	20
60	60	40	45.00	80	20
60.02	35	65	45.01	25	75
70	35	65	55	25	75

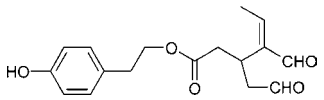

Figure 1. Chemical structure of the throat-burning oleocanthal.

solution was prepared by dilution from the stock solution in methanol/water (1/1, v/v). The working solution was used to spike olive oil samples or to prepare calibration solutions with or without further dilution with methanol/water.

Spiking Oleocanthal and/or 3,5-Dimethoxyphenol into Corn Oil or Olive Oil. For recovery determination, establishment of calibration curves, and determination of oleocanthal by calibration to an ISTD, certain volumes of standard solution or its dilution were pipetted into a centrifuge tube containing 1 g of preweighed corn oil or olive oil. Prior to solvent extraction, the contents in the centrifuge tube were mixed thoroughly by vortex for 15 s twice to allow homogeneous distribution of the standards in oil. For establishment of external calibration curves, oleocanthal was added to corn oil (1 g) at 1, 2, 5.6, 28, 140, and 700 μg/g levels. For establishment of an internal calibration curve, aliquots of corn oil (1 g) were spiked with oleocanthal at the same concentration (20 μg/g) and with increasing concentrations of ISTD 3,5-dimethoxyphenol (1, 5, 10, 20, 50, 100, 250, and 500 μg/g). For recovery determination, corn oil (1 g) was spiked with oleocanthal at a 140 or 280 μg/g concentration. To quantify oleocanthal using internal calibration, ISTD was spiked to olive oil (1 g) at a concentration of 50 μg/g.

Extraction of Oleocanthal from Oil. Oleocanthal was extracted from the oil matrix by liquid−liquid partitioning according to the following procedure. Hexane (2 mL) was added to a 15 mL centrifuge tube containing 1 g of corn oil or extra virgin olive oil. The tube was vortexed for 15 s twice to mix oil with hexane thoroughly. Five milliliters of extraction solvent (acetonitrile, acetonitrile−water mixtures, or methanol) was added, followed by vortexing the tube twice for 15 s. The tube was centrifuged at 4000 rpm for 5 min to separate the solvent from the oil phase, and the solvent extract was collected in another centrifuge tube. Each sample was extracted three times in this way, and the solvent of the combined extract was removed with a N_2 stream. One milliliter of methanol/water (1/1, v/v) was pipetted into the tube to dissolve the residue of the extract. Hexane (3 mL) was added to the solution to wash away any remaining oil. The tube was centrifuged for phase separation, and the methanol/water phase was collected for HPLC analysis. In trials without using hexane, solvent extraction was directly performed on oil.

HPLC Conditions. An Agilent 1100 series HPLC system with UV detector set to 278 nm was used. Separation was performed on a Phenomenex Luna C18(2) column (250 mm × 4.6 mm, 5 μm; Phenomenex Inc., Torrance, CA) at 25 °C. Elution was performed with two gradients using different solvents (**Table 1**). Gradient 1 was adopted from our previous work (unpublished), and gradient 2 was developed to improve separation and to reduce run time. The flow rate was 1 mL/min, and the injection volume was 20 μL.

RESULTS AND DISCUSSION

Comparison of Extraction Solvents. As shown in **Figure 1**, oleocanthal is a phenolic compound. The phenolic compounds of olive oils are generally isolated by extraction of an oil solution in hexane with several portions of water/methanol, followed by solvent evaporation of the aqueous extract and a cleanup of the residue by solvent partition (*5*, *6*). Attempts have also been made to isolate phenolic compounds by solid-phase extraction using C18 (*7*, *8*), C8 (*9*, *10*), or diol-phase cartridges (*11*).

Table 2. Recovery (%) of Oleocanthal from Corn Oil When Extracted with Different Solvents

	extraction solvent volume (mL)				
trial no.	hexane	acetonitrile	methanol	water	recovery[a] (%)
1	0	0	5	0	80.3 ± 0.8
2	0	5	0	0	91.7 ± 1.4
3	2	5	0	0	96.0 ± 2.0
4	2	4.5	0	0.5	71.8 ± 1.9
5	2	3.75	0	1.25	76.9 ± 0.8

[a] Average of duplicate experiments; each sample was extracted three times with the specific solvent system.

In this work, solvent extraction was optimized and used to isolate oleocanthal with other phenols from olive oils. Oleocanthal was spiked into about 1 g of corn oil at a 140 ppm level and then extracted with different solvents as described in the Materials and Methods. The oleocanthal extract was analyzed by HPLC, and recoveries were calculated based on peak areas. As shown in **Table 2**, recovery of oleocanthal from corn oil when extracted with methanol three times was only 80.3%, which was far from quantitative. When methanol was replaced with acetonitrile, the recovery increased significantly to 91.7%. Therefore, acetonitrile was preferred in the extraction of oleocanthal from oil matrices. On the other hand, it had been reported that the addition of specific solvents (hexane, petroleum ether, or chloroform) to the oils did not increase the phenolic concentration of the extracts (*5*). However, in this work, dissolution of oil in hexane prior to extraction with acetonitrile further increased the recovery of oleocanthal from 91.7 to 96.0% (**Table 2**). The addition of water to acetonitrile reduced the recovery of oleocanthal from corn oil as shown in **Table 2**.

We observed later that oleocanthal, either in standard solutions or when extracted from olive oil, disappeared instantaneously in acetonitrile/water (1/1,v/v) solution. However, oleocanthal appeared to be quite stable in pure acetonitrile. It is known that phenolic compounds degrade rapidly under alkaline conditions. Catalan et al. (*12*) characterized acetonitrile/water binary solvent mixtures in terms of their polarity, acidity, and basicity. They found that the basicity of acetonitrile solvent mixture rises gradually as water is added to acetonitrile and further water addition causes it to drop to a virtually negligible value for water. According to their work, the basicity of acetonitrile/water (v/v) mixtures (1/1, 3/1, and 9/1) was higher than that of pure acetonitrile, while the basicity of acetonitrile/water (v/v, 1/3) was almost identical to that of acetonitrile. This could explain the instability of oleocanthal in acetonitrile/water (1/1,v/v) solvent while relatively stable in acetonitrile, the lower recoveries of oleocanthal when water was added to acetonitrile (**Table 2**), and the stability of oleocanthal during the chromatographic process with isocratic acetonitrile/water (v/v, 1/3) as the mobile phase. A further simple stability study showed that oleocanthal was stable in methanol/water (v/v, 1/1) with or without 0.5% HOAc for at least 24 h.

It is worthwhile to mention here that recoveries of oleocanthal were lower, irregular, and irreproducible when it was spiked into a previously opened (more than 1 year) bottle of Bertolli pure olive oil and extracted with the same solvents according to the same procedure. It was likely that compounds formed

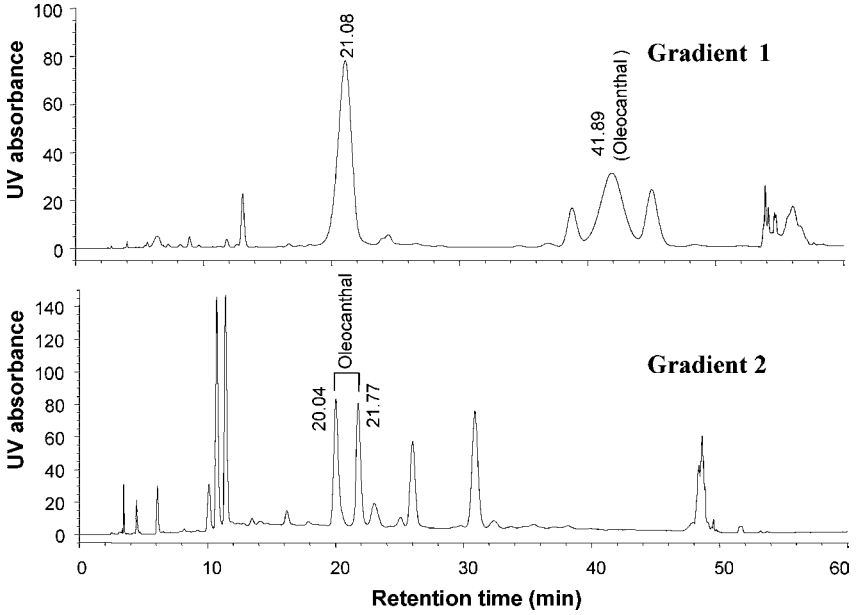

Figure 2. Comparison of HPLC chromatograms of olive oil phenolic compounds obtained using two mobile phase systems.

during the degradation of the pure olive oil, such as free fatty acids and peroxides, in this old sample of pure olive oil could have caused the degradation of oleocanthal.

HPLC Separation of Olive Oil Phenolic Compounds. Currently, analysis of phenolic compounds is performed by reversed-phase HPLC with UV detection operated at 225, 240, or 280 nm, using gradient elution with two solvents, one of them a water/acid mixture, and the other acetonitrile, methanol or methanol/acetonitrile. A fraction of phenolic extract containing oleocanthal was analyzed by HPLC using gradient 1 as the mobile phase (**Table 1**). The resulting chromatogram (top) is shown in **Figure 2**. Injection of oleocanthal standard was used to identify its peak in the chromatogram. Oleocanthal was eluted at 41.89 min as a broad peak, most likely due to equilibrium with its methoxy hemiacetal during the HPLC analysis (*11*). In fact, in NMR studies of oleocanthal, Montedoro (*2*) observed a reversible equilibrium between oleocanthal and its hemiacetal derivative. To shorten the retention time and to improve the separation of oleocanthal from the two adjacent phenolic peaks, an HPLC mobile phase consisting of acetonitrile and water (gradient 2) was developed for the separation of oleocanthal from other olive oil phenols. Interestingly, this gradient resulted in much more effective separation of olive oil phenols as shown in **Figure 2**. Oleocanthal was separated into two sharp peaks that eluted at 20.04 and 21.77 min. The two peaks likely represent the two geometric isomers (cis and trans at the double bond) of oleocanthal. Therefore, gradient 2 was used for the rest of this study. Because of the instability of oleocanthal in acetonitrile/water mixture as discussed in the previous section, samples containing oleocanthal for HPLC analysis were prepared in methanol/water (1/1, v/v) solvent mixture as stated in the Materials and Methods.

Calibration with External Standard. Quantitation of oleocanthal can be performed with external calibration if a pure standard is available. As shown in **Table 2**, when oleocanthal was spiked into corn oil at 140 ppm level and the spiked oil was first dissolved in hexane and then extracted with acetonitrile, 96% of oleocanthal was recovered. However, it was not known whether oleocanthal could be recovered at the same rate when spiked at lower or higher levels. Therefore, oleocanthal was spiked into about 1 g of corn oil at levels that varied from 1 to 700 ppm. Oleocanthal was then extracted and analyzed by HPLC, and the concentrations of oleocanthal were plotted against its peak areas to establish a calibration curve. As shown in **Figure 3**, the calibration curve shows good linearity over the range examined (1−700 ppm). Therefore, oleocanthal can be accurately quantified to as low as 1 ppm in oil. Accurate quantification of oleocanthal to a lower level using this method may be possible.

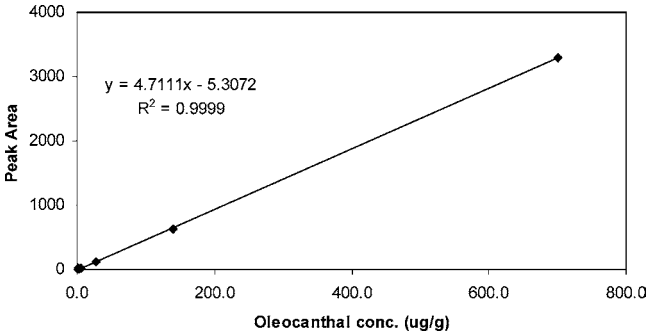

Figure 3. Calibration curve of oleocanthal obtained by spiking it into corn oil and its linearity.

Calibration with ISTD. Oleocanthal is not currently commercially available, and it is tedious to either isolate it from extra virgin olive oil or synthesize it (*4*). Furthermore, the compound does not seem to be stable for a long period of time. Internal calibration using another stable compound would save much effort, in which case only small amounts of oleocanthal would be required once to establish a calibration curve. An additional advantage of internal calibration is that errors caused by inaccurate volume transfer are compensated. After several screening experiments, 3,5-dimethoxyphenol was chosen as the ISTD because of its UV absorbance at 278 nm and its chromatographic retention. The calibration curve was established by spiking 20 ppm of oleocanthal into seven samples of corn oil (1 g), along with increasing concentrations of 3,5-dimethoxyphenol (ISTD) (5, 10, 20, 50, 100, 250, and 500 ppm). Oleocanthal and ISTD were then extracted and analyzed by HPLC, and the ratios of the oleocanthal peak area over the ISTD peak area were plotted against the ratios of oleocanthal concentration over ISTD concentation, resulting in the linear calibration curve shown in **Figure 4**.

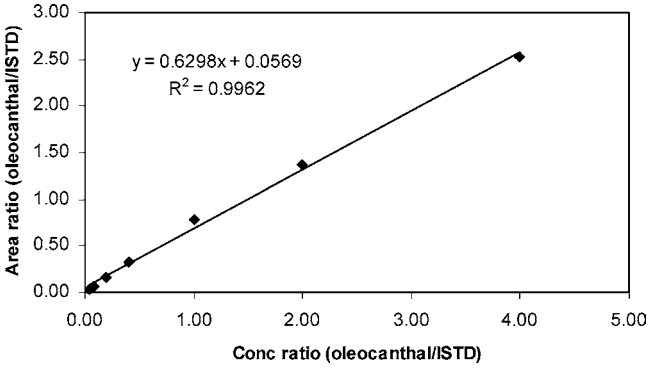

Figure 4. Linearity of oleocanthal calibration curve with 3,5-dimethoxyphenol as an ISTD by spiking both into corn oil.

Table 3. Recoveries (%) of Oleocanthal When Spiked to an Extra Virgin Olive Oil (Falconero) that Contained the Compound Itself

falconero oil (g)	amount spiked (μg)	concentration (μg/g) determined	calculated amount (μg) of spiked	recovery (%)
1.0100	0	188.1	NA	NA
1.0138	140	327.3	141.1	100.8
1.0072	140	322.2	135.1	96.5
1.0057	280	453.8	267.2	95.4
1.0056	280	459.5	272.9	97.5

Reproducibility and Accuracy. One of the olive oil samples (Falconero) was analyzed in triplicate, and peak areas of oleocanthal per gram of oil were calculated. Relative standard deviation (RSD) of the peak areas was 4.7%, indicating that the reproducibility of the method was good. The accuracy of the method was also checked by spiking experiments. Known amounts of oleocanthal were spiked into about 1 g of Falconero olive oil samples. The oleocanthal in the spiked oil samples and in the original oil sample was extracted and analyzed by HPLC. Concentrations of oleocanthal in the samples were determined using the calibration curve shown in **Figure 3**. The amounts of oleocanthal spiked into the samples were then calculated by subtracting the amounts of oleocanthal present in the original oil from the total amounts of oleocanthal determined in the spiked samples. The recoveries of oleocanthal in the four spiked samples were then obtained (**Table 3**). Good recoveries of oleocanthal suggest good accuracy of the method. In one olive oil sample, ISTD was spiked at 50 ppm, and the level of oleocanthal was calculated using both calibration curves (**Figures 3** and **4**). The values obtained (49.4 ppm with external calibration and 51.1 ppm using internal calibration) were essentially the same within the standard deviation.

Determination of Oleocanthal in Throat-Burning Extra Virgin Olive Oils. Ten selected throat-burning extra virgin olive oils were analyzed in duplicate using the HPLC method. The concentrations of oleocanthal were calculated using the calibration curve shown in **Figure 3**. The results are listed in **Table 4** in increasing order of the concentration of oleocanthal. In general, the relative standard deviations of duplicate analyses are within 5%, except for Caroli oil from Italy. Olio Santo oil from California had the lowest level of oleocanthal, while Falconero oil from Italy had the highest level (189.9 μg/g) of oleocanthal.

In conclusion, a simple reversed-phase HPLC method using UV detection at 278 nm was developed to quantitatively determine recently discovered natural antiinflammatory oleocanthal in olive oil. The extraction procedure was tailored to avoid the degradation of oleocanthal and for high extraction efficiency. Quantitation using external standard calibration or ISTD calibration gave the same result. The sample preparation procedure and the HPLC conditions can be used for HPLC-MS analysis of oleocanthal in olive oil to improve sensitivity and selectivity. The concentrations of oleocanthal in 10 selected throat-burning extra virgin olive oils were determined using the method.

Table 4. Concentrations (μg/g) of Oleocanthal in the 10 Extra Virgin Olive Oils

	concentration (μg/g)				
olive oil (origin)	analysis 1	analysis 2	average	SD	RSD (%)
Olio Santo (California)	22.1	23.0	22.6	0.6	2.81
Sitia (Crete)	36.6	37.5	37.1	0.6	1.70
Caroli (Italy)	39.9	43.9	41.9	2.8	6.65
Lucini (Italy)	42.1	44.1	43.1	1.4	3.33
Horio (Minerva, Greece)	49.8	48.3	49.1	1.0	2.07
Spitiko (Volos, Greece)	53.5	50.2	51.8	2.4	4.57
Colonna (Italy)	85.6	81.2	83.4	3.1	3.75
Frantoio di Santa Tea (Italy)	129.3	132.2	130.7	2.1	1.57
Laudemio (Italy)	154.2	153.3	153.7	0.6	0.41
Falconero (Italy)	191.8	188.1	189.9	2.7	1.40

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