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## Influence of Heat on Biological Activity and Concentration of Oleocanthal—a Natural Anti-inflammatory Agent in Virgin Olive Oil

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The olive oil phenolic oleocanthal is a natural nonsteroidal anti-inflammatory compound that irritates the oral pharynx in a dose-dependent manner. It has been proposed that the biological activity of oleocanthal is partially responsible for the beneficial health effects of the Mediterranean diet. Virgin olive oil containing oleocanthal is often added as an ingredient in a number of cooked dishes, and therefore it is of great importance to understand how best to preserve the putative health-promoting benefits of this compound, as olive oil phenolics are subject to degradation upon heating in general. One extra virgin olive oil containing 53.9 mg/kg oleocanthal was heated at various temperatures (100, 170, and 240 °C) for set time periods (0, 1, 5, 20, 60, and 90 min). Oleocanthal concentrations were quantified using HPLC, and its biological activity was determined with a taste bioassay measuring the intensity of throat irritation. Results demonstrated that oleocanthal was heat stable compared with other olive oil phenolics, with a maximum loss of 16% as determined by HPLC analysis. However, there was a significant decrease of up to 31% ( $p < 0.05$ ) in the biological activity of oleocanthal as determined by the taste bioassay. Although there was minimal degradation of oleocanthal concentration, there was a significant decrease in the biological activity of oleocanthal upon extended heating time, indicating a possible loss of the putative health-benefiting properties of oleocanthal. Alternatively, the difference in the concentration and biological activity of oleocanthal after heat treatment could be a result of an oleocanthal antagonist forming, decreasing or masking the biological activity of oleocanthal.

**KEYWORDS:** Olive oil; HPLC; sensory; Mediterranean diet; health benefits

### INTRODUCTION

Historically, the healthful properties of virgin olive oil were attributed to a high proportion of monounsaturated fatty acids (MUFAs), namely, oleic acid, which represents 70–80% of the fatty acids present in virgin olive oil (1). However, several seed oils (including sunflower, soybean, and rapeseed) rich in MUFA have been demonstrated to be ineffective in beneficially altering chronic disease risk factors (2, 3). In addition to MUFA, virgin olive oil contains a minor, yet significant, phenolic component that other seed oils lack. Thus, the phenolic fraction of virgin olive oil has generated much interest regarding its health-promoting properties. Subsequent studies (human, animal, in

vivo, and in vitro) have demonstrated that olive oil phenolics have positive effects on certain physiological parameters, possibly reducing the risk of chronic disease development (for a review see ref 4).

One such olive oil phenolic of particular interest due to its putative health-benefiting properties is decarboxymethyl ligstroside aglycone (**Figure 1**). Decarboxymethyl ligstroside aglycone was first identified in virgin olive oil by Montedoro et al. in 1993 (5). In 2003, Andrewes and co-workers (6) independently reported that decarboxymethyl ligstroside aglycone was responsible for the throat irritation associated with some extra virgin olive oils. In 2005, Beauchamp et al. (7), using a taste bioassay, isolated oleocanthal from virgin olive oil and measured its intensity of throat irritation. The intensity of throat irritation was dose-dependent on oleocanthal concentration. To exclude the thought that coelution of a minor component or a mixture of components could be the cause of throat irritation, the authors chemically synthesized oleocanthal and dissolved it in nonirritating corn oil. Throat irritation elicited by synthesized oleocanthal was dose-dependent on the concen-

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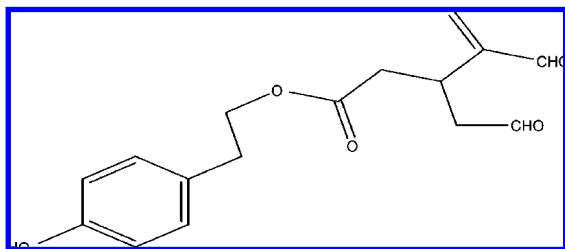


Figure 1. Chemical structure of oleocanthal.

tration of oleocanthal in corn oil and mimicked that of virgin olive oil containing oleocanthal. Thus, oleocanthal was deemed to be the sole throat irritant in virgin olive oil. Furthermore, the authors reported that decarboxymethyl ligstroside aglycone was a natural NSAID present in virgin olive oils and named the compound oleocanthal (oleo = olive, canth = sting, and al = aldehyde).

Oleocanthal, which has not been identified in any other vegetable oil, is responsible for the stinging sensation localized to the posterior oropharyngeal region upon virgin olive oil consumption (7). This sensation has been described as a peppery bite at the back of the throat (7) and is similar to that caused by the NSAID ibuprofen (8). This finding provoked the hypothesis that oleocanthal might possess pharmacological properties similar to those of ibuprofen and several other NSAIDs (7). Beauchamp and colleagues (7) demonstrated that oleocanthal did indeed possess anti-inflammatory activity due to its dose-dependent ability to inhibit the same cyclooxygenase (COX) enzymes in the prostaglandin biosynthesis (inflammatory) pathway as ibuprofen. Interestingly, it has also been demonstrated that the dose-dependent anti-inflammatory properties oleocanthal exhibits *in vitro* are mimicked by its dose-dependent irritation in the oral cavity (7), making the taste bioassay a *de facto* marker of biological activity (9, 10). Furthermore, it is important to note that long-term ingestion of small chronic doses of oleocanthal via virgin olive oil consumption may be responsible, in part, for the low incidence of heart disease, certain cancers, and other degenerative diseases associated with the Mediterranean diet (7).

During normal cooking procedures, virgin olive oil temperatures can range from 170 to 190 °C and can be used up to 6 to 10 times in deep and shallow frying (11). In general, olive oil phenolics are subject to degradation upon the application of heat during cooking, although this loss appears to vary among the different phenolic compounds (11–13). To date, only one study has examined the effect heat has on the degradation of oleocanthal (12). This study observed a 20% decomposition of oleocanthal after 2 h of frying at 180 °C. Further studies are therefore required to build a body of knowledge regarding the heat stability of this compound.

A number of methods including Folin–Ciocalteu and high-performance liquid chromatography (HPLC) have been used in the identification and quantification of phenolic compounds in virgin olive oil. Although the Folin–Ciocalteu method is economical, a limitation to this method is its low specificity and its inability to provide qualitative information on any individual olive oil phenolic. On the other hand, HPLC is sensitive and specific and, when accompanied by standards or a qualitative method such as mass spectrometry (MS), it can provide quantitative and qualitative information on phenolic compounds present in virgin olive oil (1, 14). Thus, a HPLC method after liquid–liquid extraction has been developed to quantitatively analyze oleocanthal in virgin olive oil (15).

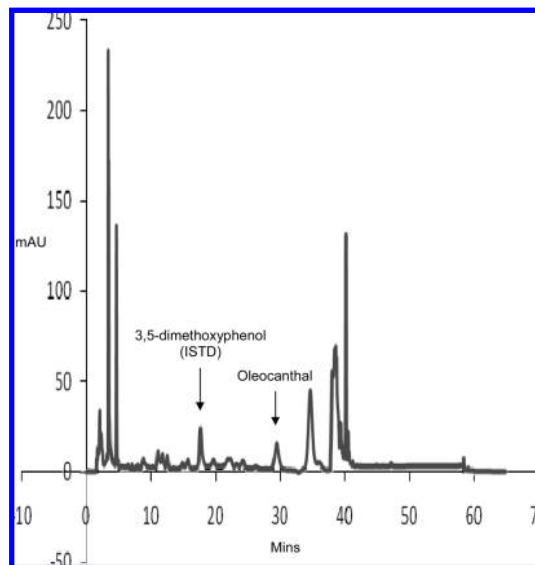


Figure 2. HPLC chromatogram of olive oil phenolic extract, showing the peaks of oleocanthal and 3,5-dimethoxyphenol.

Overall, it is of great importance to determine the heat stability of oleocanthal, as virgin olive oils containing this compound are often consumed after the application of heat. Use of both HPLC and bioassay analysis will aid in not only determining the concentration of oleocanthal after heating but also examining the effect of heating on the biological activity of oleocanthal. This is of great importance, as physiological properties of particular compounds have been related back to their sensory perception (9, 10), particularly in the case of oleocanthal (7). Through this knowledge the putative health-promoting benefits of oleocanthal may be better preserved. Hence, the aim of this research was to investigate if oleocanthal is thermally degraded or its biological activity reduced using normal cooking times and temperatures.

## MATERIALS AND METHODS

**Oil Samples.** One extra virgin olive oil (Red Island Australia, Malaga, Australia) containing 53.9 mg/kg of oleocanthal was heated to 100, 170, and 240 °C for 0, 1, 5, 20, 60, and 90 min, using a full-factorial design. Oleocanthal concentration was determined by calculating the ratio of oleocanthal peak area to 3,5-dimethoxyphenol (internal standard, ISTD) peak area and multiplied by the amount of ISTD added to the sample. A positive control (un-heat-treated extra virgin olive oil) and a negative control (corn oil, devoid in oleocanthal) (Nature First, Cheltenham, Australia) were also included in the analysis.

**HPLC Analysis.** The method used for HPLC analysis of oleocanthal was modified from the method developed by Impellizzeri and Lin (15). Refer to **Figure 2** for a typical HPLC chromatogram. Samples were prepared and analyzed in triplicate.

**Standard Solution.** A stock solution (5000 mg/kg) of the ISTD 3,5-dimethoxyphenol (Aldrich) was prepared in methanol (Rowe Scientific, Hallam, Australia). This solution was used to spike the oil samples.

**Addition of 3,5-Dimethoxyphenol to Oil.** To quantify oleocanthal, the ISTD was added to extra virgin olive oil (10 g) at a concentration of 500 µg/g. Oleocanthal concentration was expressed as ISTD equivalents.

**Extraction of Oleocanthal from Oil.** Oleocanthal was extracted from the oil matrix by liquid–liquid partitioning according to the following procedure. Ten milliliters of hexane (Rowe Scientific) was added to a centrifuge tube containing 10 g of oil. The tube was vortexed for 1 min to mix oil and hexane thoroughly. Fifty milliliters of acetonitrile (Rowe Scientific) (extraction solvent) was added and then vortexed for 1 min. The tube was centrifuged at 4000 rpm for 5 min for separation of the solvent from the oil phase. The solvent extract was collected and placed in an evaporator flask, and the solvent was removed with

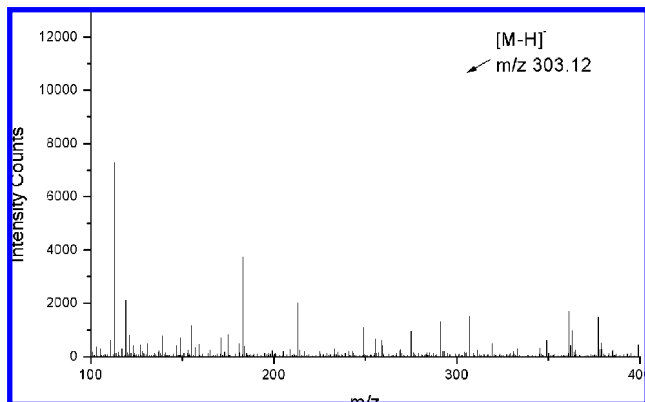


Figure 3. Negative ion mass spectrum of oleocanthal.

the use of a rotary evaporator (In Vitro Technologies, Noble Park North, Australia) at 45 °C and 200 mbar. One milliliter of methanol/water (HPLC grade) (1:1, v/v) was pipetted into the flask containing the dried extract to dissolve the residue of this extract. The extract dissolved in methanol/water was then transferred to a centrifuge tube. One milliliter of hexane was added to the solution to aid in the removal of any remaining oil. The tube was centrifuged (4000 rpm for 2 min) for phase separation. The methanol/water phase was collected for HPLC analysis.

**HPLC Conditions.** Oleocanthal was separated using a 1200 series HPLC system with solvent degasser, quaternary pump, autosampler, and diode array detector set to 278 nm (Agilent Technologies, Blackburn, Australia). An Apollo RP-C18 column (250 mm × 4.6 mm i.d., 5 μm; Grace Davison, Baulkham Hills, Australia) was used for all separations at a constant temperature of 25 °C using the gradients of 75% water and 25% acetonitrile for the first 35 min of the run, then 90% acetonitrile and 10% methanol for a further 20 min, and then back to the initial gradient of 75% water and 25% acetonitrile for the remaining 10 min of the run. The flow rate was 1 mL/min, and the injection volume was 20 μL.

**Mass Spectrometry (MS) Conditions.** Oleocanthal was identified using a 6210 MSDTOF mass spectrometer (Agilent Technologies, Blackburn, Australia) under the following conditions: drying gas, nitrogen (7 mL<sup>-1</sup>, 350 °C); nebulizer gas, nitrogen (15 psi); capillary voltage 4.0 kV; vaporizer temperature, 350 °C; and cone voltage, 60 V. **Figure 3** displays the negative ion mass spectrum of oleocanthal with the characteristic [M-H]<sup>-</sup> ion at *m/z* 303.12 highlighted.

**Biological Activity Assay.** The method used for the oleocanthal biological activity assay was modified from the taste bioassay protocol developed by Beauchamp et al. (7). Oleocanthal elicits irritation in the oral pharynx in a dose-dependent manner, mirroring COX inhibitory activity, meaning biological activity can be determined via a taste bioassay. Each subject was tested a maximum of two times per day with two different oil samples. Test sessions were scheduled so that there was at least 2 h between each test. Each sample was evaluated in triplicate.

**Subjects.** Subjects (*n* = 10, 28.3 ± 8.0 years, 9 female) between the ages of 21 and 42 years were university staff and students in Melbourne, Australia. All subjects agreed to participate in the study and provided informed consent on an Institutional Review Board form approved by the Deakin University Ethics Committee. The participants were asked to refrain from consuming food and drink (except room temperature water) and using oral irritants (such as toothpaste, mouthwash, and gum) 2 h prior to testing.

**Subject Training.** Participants were trained in the use of the general Labeled Magnitude Scale (gLMS) following the published standard procedures of Bartoshuk et al. (16). The gLMS is a labeled scale of sensation intensity that requires participants to rate perceived intensity along a vertical axis containing the following adjectives: barely detectable = 1.5, weak = 6, moderate = 17, strong = 35, very strong = 52, and strongest imaginable = 100. These adjectives are placed semilogarithmically and are derived from experimentally determined intervals that yield data equivalent to magnitude estimation (16–18). The adjectives, but not their corresponding numbers, are visible only to the participant. The experimenter calculates numerical data from

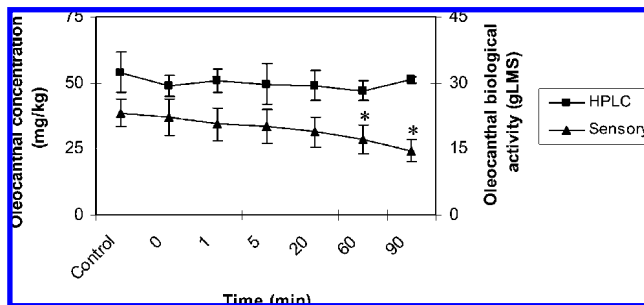


Figure 4. Oleocanthal concentration (ISTD equivalents) quantified by HPLC (mean ± SD) and biological activity as determined by the taste bioassay (mean ± SE) at 100 °C. \*, significant difference (*p* < 0.05) observed between control oil and heat-treated oil.

the scale. Participants attended a training session to evaluate oleocanthal irritation intensity with the use of an extra virgin olive oil containing oleocanthal.

**Stimuli and Delivery.** Oils were stored at 4 °C under oxygen-free conditions (N<sub>2</sub> purging was used to maintain samples in this way) and brought to room temperature (20 ± 3 °C) prior to testing. All testing took place in a specialized testing facility comprising seven individual computerized booths, containing red lights to mask color differences in the oils. Each subject was isolated from other subjects by vertical dividers to eliminate interaction between subjects (19).

**Stimulus delivery** was adapted from the methods of Beauchamp et al. (7). An aliquot of 5 mL of each oil (*n* = 20) was presented in 30-mL polyethylene medicine cups (McFarlane Medical, Surrey Hills, Australia) in a fully randomized order. Subjects rinsed their mouths with filtered (fi) water (8 μm particulate filter with an activated charcoal filter, Dura) at least three times over a 2-min period before commencement of testing. Subjects wore nose clips to eliminate olfactory cues. Each subject swallowed the oil in two aliquots, and after 20 s had elapsed, subjects were asked to rate (with use of the gLMS) the oils for intensity of peak throat irritation.

**Statistical Analyses.** Statistical comparison of means was investigated with both paired *t* test and two-way ANOVA (SPSS 14.0, Chicago, IL); *p* values of <0.05 were considered to be significant. A Pearson product-moment correlation was also conducted to investigate the relationship between the HPLC quantified concentration and the biological activity of oleocanthal in the extra virgin olive oil samples.

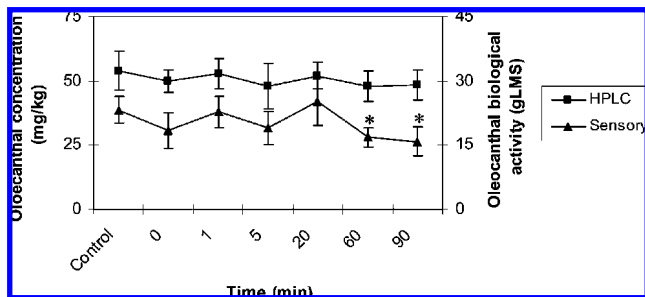
## RESULTS AND DISCUSSION

**Chemical Stability of Oleocanthal upon Heating.** Oleocanthal concentration was quantified using HPLC; in addition, both the oleocanthal and ISTD peaks were identified using MS and retention time data of oleocanthal standard from previous papers (15). In this instance, the quantification of oleocanthal was performed by reference to an internal standard, 3,5-dimethoxyphenol, which has previously been shown as a suitable molecule for oleocanthal quantification (15).

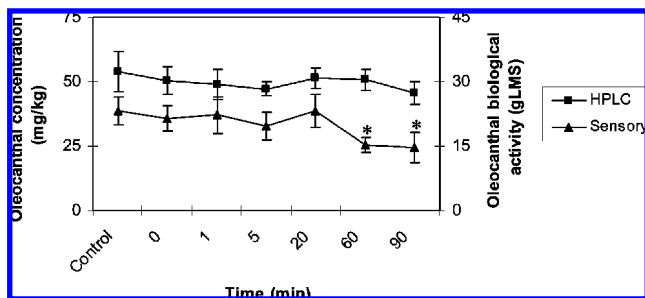
A two-way ANOVA revealed there was no main effect of time [*F*(6, 42) = 1.2, *p* = 0.3] or temperature [*F*(2, 42) = 0.05, *p* = 0.9] on oleocanthal concentration. For instance, the concentration of oleocanthal over 90 min of heating at 100, 170, and 240 °C was fairly stable at 51.1 ± 1.4, 48.3 ± 5.9, and 45.5 ± 4.3 mg/kg, respectively (refer to **Figures 4–6**).

After heat treatment at 240 °C for 90 min, minimal degradation in oleocanthal was observed (53.9 ± 7.7 to 45.5 ± 4.3 mg/kg, equating to a 16% decrease), supporting an earlier finding showing a similar rate of decomposition (20%) after 2 h of frying at 180 °C (12). However, a significant decrease in concentration after treatment at 240 °C for 90 min was observed in the phenolics: hydroxytyrosol (63.5 ± 8.0 to 16.4 ± 8.0 mg/kg, equating to a 74% decrease, *p* < 0.05), oleuropein (20.9 ± 2.9 to 5.4 ± 0.3 mg/kg, equating to a 74% decrease, *p* < 0.05),





**Figure 5.** Oleocanthal concentration (ISTD equivalents) quantified by HPLC (mean  $\pm$  SD) and biological activity as determined by the taste bioassay (mean  $\pm$  SE) at 170 °C. \*, significant difference ( $p < 0.05$ ) observed between control oil and heat-treated oil.



**Figure 6.** Oleocanthal concentration (ISTD equivalents) quantified by HPLC (mean  $\pm$  SD) and biological activity as determined by the taste bioassay (mean  $\pm$  SE) at 240 °C. \*, significant difference ( $p < 0.05$ ) observed between control oil and heat-treated oil.

and oleuropein aglycone ( $22.2 \pm 7.3$  to  $9.2 \pm 2.6$  mg/kg, equating to a 59% decrease,  $p < 0.05$ ). These results were also in agreement with earlier results demonstrating hydroxytyrosol and oleuropein aglycon decreased by 90–100% after 2 h of frying at 180 °C (12). The minimal degradation of oleocanthal in comparison to other phenolics measured may be partially due to the compound's chemical structure and subsequent antioxidant activity.

The antioxidant capacity of phenolic compounds varies and is dependent upon a number of factors including the number of hydroxyl groups bonded to the aromatic ring (20). When free radicals are produced through oxidation, phenolic compounds with a higher number of hydroxyls and, therefore, increased antioxidant capacity quickly diminish because they act by reacting rapidly with lipid radicals and are therefore consumed (12, 20). Oleocanthal possesses one hydroxyl group, whereas hydroxytyrosol, oleuropein, and oleuropein aglycone contain three, six, and three hydroxyl groups, respectively. A strong correlation ( $r = 0.9$ ) was observed between phenolic degradation and the number of hydroxyls contained by the phenolics examined in this study. Furthermore, a correlation was conducted by the current authors with the data obtained by Gomez-Alonzo et al. (12), and a strong correlation ( $r = 0.7$ ) was also found between the concentration of phenolics quantified after 12 frying operations and the number of hydroxyl groups. This concept may explain why there was minimal degradation of oleocanthal compared to the additional three phenolics investigated in this study.

The site of bonding and mutual position of hydroxyls in the aromatic ring have also been postulated to play roles in the antioxidant potential of phenolics (20, 21). For instance, Rice-Evans et al. (21) demonstrated that a hydroxyl group in the ortho position in the aromatic ring results in increased antioxidant capacity compared to compounds with hydroxyl groups in the

meta and para positions. Oleocanthal contains its one hydroxyl group in the para position, whereas hydroxytyrosol, oleuropein, and oleuropein aglycone contain their hydroxyl groups in the ortho position. This theory may also help in explaining differences in heat degradation among olive oil phenolics.

#### Stability of Oleocanthal Biological Activity upon Heating.

The biological activity assay is a measure of oleocanthal irritation in the oral pharynx. Increased intensity of oral pharyngeal irritation denotes higher biological activity of oleocanthal.

A two-way ANOVA revealed there was no main effect of temperature [ $F(2, 42) = 0.3$ ,  $p = 0.7$ ] but there was a significant main effect for heating time regarding the biological activity of oleocanthal [ $F(6, 42) = 6.8$ ,  $p < 0.005$ ]. No significant decrease ( $p = 0.3$ – $1.0$ ) in oleocanthal biological activity was observed for up to 20 min. However, by 60 min of heating there was a significant decrease ( $p < 0.05$ ) in oleocanthal biological activity (refer to **Figures 4–6**). Percentage decreases in biological activity for the 60 and 90 min samples were 31% and 28%, respectively.

It appears that after 20 min there is an unknown factor that significantly inhibits the biological activity of oleocanthal, as we know from analytical data the degradation of oleocanthal is not significant (with an absolute degradation of 8.4 mg/kg, equating to a 16% decomposition). A possible explanation is that during the heating process a new compound (an oleocanthal antagonist) may form, decreasing or masking the biological activity of oleocanthal. Another explanation for this finding is that the human sensory system may be sensitive to a modest decrease in oleocanthal concentration. In support of this, the oral dose–response curve of compounds ( $\Psi = k\Phi^n$ ) is rarely a 1:1 ratio or linear relationship (22). When  $n$  is  $> 1$ , the sensation grows more rapidly than the stimulus, and when  $n$  is  $< 1$ , the sensation grows more slowly. For example, the exponents of oral activation by quinine–HCl and NaCl are 0.65 and 1.4, respectively. This simply means that an increase of 1 mM quinine–HCl would result in a 0.65 increase in oral perception of quinine–HCl, whereas an increase of 1 mM of NaCl would result in a 1.4 increase in oral perception of NaCl (22). Also, although it is highly unlikely given previously published data (7), we should not discount the possibility that other phenolic compounds may have a synergistic effect on the oropharyngeal irritation and that heat degradation of those compounds may result in decreased irritation.

The taste bioassay has shown the biological activity of oleocanthal is affected by extended heating, possibly due to an antagonist in the olive oil matrix; however, this requires further investigation. The combination of HPLC and the taste bioassay allows investigators to not only examine the physical result of heating oleocanthal (i.e., concentration) but also study the effect heating may have on the biological activity of oleocanthal in humans. In support of this research, it is important to note that the physiological functions of particular compounds have been related back to their perceived oral intensity (7, 9, 10). Additionally, correlation analysis demonstrated that a strong positive correlation ( $r = 0.8$ ,  $p < 0.05$ ) existed between the HPLC analysis of concentration and the biological activity of oleocanthal. Therefore, the taste bioassay proves to be an effective tool in analyzing the biological activity of oleocanthal (7).

**Retention of Oleocanthal upon Heating and Implications for Health.** The heat stability of oleocanthal has important implications for health. Although the concentration of oleocanthal remained constant upon heating, there was a significant

decrease in the biological activity of oleocanthal with extended heating time. A significant decrease (up to 31% at 90 min heating) in the biological activity of oleocanthal may indicate that a similar proportion of the putative health-benefiting properties of oleocanthal may be lost during extended cooking. Inflammation has been demonstrated to play a significant role in a number of chronic diseases. Therefore, it has been suggested that long-term ingestion of small chronic doses of oleocanthal via virgin olive oil consumption may be responsible, in part, for the low incidence of degenerative diseases associated with the Mediterranean diet (7). Further studies are required to determine if the decrease in oral biological activity is mirrored by a decrease in COX enzyme inhibition.

In conclusion, minimal degradation of oleocanthal concentration was observed upon heating (16%); however, a significant decrease in the biological activity of this compound was noted with extended heating time (up to 31%). This has important implications for health in that consumers may be unable to reap all of the putative health benefits associated with this phenolic compound when adding oleocanthal-containing virgin olive oil as an ingredient to dishes requiring prolonged heat treatment. Alternatively, the reduction in biological activity after heating may be due to the formation of an oleocanthal antagonist, which may mask the biological activity but not necessarily reduce the concentration of oleocanthal. Further studies are therefore required to establish if the decrease in oral biological activity is mirrored by a decrease in COX enzyme inhibition.

#### ACKNOWLEDGMENT

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