ANALYTICAL LABORATORY REPORT

Qualitative and Quantitative Analysis of Biophenols, Physicochemical Quality Indices and EFSA Health Claim Verification

Extra Virgin Olive Oil — Organic

REPORT IDENTIFICATION

Report No.	PHOL-2025-OO-00261
Date of Issue	30 August 2025
Analytical Method	HPLC-DAD / LC-MS Profiling | IOC Official Methods
Laboratory	Ask Farms Tarım Sanayi ve Ticaret A.Ş. — Analytical Department
Document Status	FINAL — Approved for Release

SAMPLE INFORMATION

Sample Code	TK#1250-KG02-HA225
Product Name	Fruity & Full Bodied — Organic Extra Virgin Olive Oil
Producer / Submitting Company	Ask Farms Tarım Sanayi ve Ticaret A.Ş.
Sample Matrix	Cold-pressed extra virgin olive oil (unfiltered, organic)
Sample Volume Received	2 × 1 kg sealed glass bottle
Date of Receipt	27 August 2025
Date of Analysis	28–29 August 2025
Date of Report	30 August 2025
Storage Conditions	Dark, ambient temperature (18–22 °C) prior to analysis

SCOPE OF ANALYSIS

This report presents the results of a comprehensive analytical investigation performed on the olive oil sample identified as TK#1250-KG02-HA225. The study encompasses five distinct analytical objectives as described below:

A. Extraction of Biophenols

Isolation of polar phenolic compounds from the olive oil matrix using a liquid-liquid solvent extraction procedure assisted by ultrasound and centrifugation, followed by membrane filtration and preparation of the clarified extract for chromatographic injection.

B. HPLC-DAD / LC-MS Analysis and Profiling

Chromatographic separation and compound identification using High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) at 280 nm, with confirmatory mass spectrometric analysis (LC-MS) for structural verification of individual phenolic constituents.

C. Quantitative Determination of Major Olive Oil Biophenols

Determination of the key phenolic constituents — Hydroxytyrosol (HT), Tyrosol (T), Oleacein (OLEA) and Oleocanthal (OLEO) — expressed in mg per kg of olive oil, in accordance with IOC methodology (COI/T.20/Doc No. 29).

D. Verification of EFSA-Approved Health Claim

Evaluation of total phenolic content expressed as mg Tyrosol equivalents per kg olive oil, to assess compliance with Regulation (EU) No 432/2012 concerning the protection of blood lipids from oxidative damage.

E. Physicochemical Quality Assessment

Determination of free fatty acidity (% oleic acid), peroxide value (meq O_2/kg), and UV spectrophotometric indices K_{232}, K_{270} and ΔK, following IOC official methods (COI/T.20/Doc No. 19 and subsequent revisions) for oxidation status and classification as Extra Virgin Olive Oil.

AIM OF STUDY

The primary objective of this analytical study is to determine the major biophenols in sample TK#1250-KG02-HA225 — namely Hydroxytyrosol, Tyrosol, Oleacein and Oleocanthal — and to verify compliance with the EFSA-approved health claim for olive oil polyphenols. Olive oil is a complex matrix of nutritionally valuable compounds. Pursuant to Regulation (EU) No 432/2012, olive oil polyphenols contribute to the protection of blood lipids from oxidative damage, provided the product contains at least 250 mg/kg of hydroxytyrosol and its derivatives (including oleuropein complex and tyrosol), and the consumer is informed that the beneficial effect is achieved with a daily intake of 20 g of olive oil.

In the present study, the sample was profiled by HPLC-DAD and LC-MS to establish the qualitative composition of the phenolic fraction, to quantify the individual major biophenols, and to determine the total biophenol content expressed as mg Tyrosol equivalent per kg olive oil. Complementary physicochemical indices were measured to confirm classification as Extra Virgin Olive Oil.

EXTRACTION AND SAMPLE PREPARATION

In order to isolate polar phenolic components from the TK#1250-KG02-HA225 olive oil sample, a liquid-liquid extraction approach assisted by ultrasound and centrifugation was applied. The procedure was performed in duplicate (n = 2) on independently prepared aliquots.

Extraction Parameter	Condition
Sample mass	2.0 g (± 0.001 g, analytical balance)
Internal standard	Syringic acid solution in methanol/water (80:20 v/v)
Extraction solvent	Methanol / Water (80:20 v/v)
Extraction procedure	Vortex mixing, followed by ultrasonic bath (15 min, room temperature)
Phase separation	Centrifugation (5 min), membrane filtration (0.22 µm PTFE)
Number of replicates	n = 2 independent extractions
Instrument calibration	External calibration with certified reference standards

HPLC-DAD / LC-MS ANALYSIS

High-Performance Liquid Chromatography coupled to Diode Array Detection (HPLC-DAD) and confirmatory liquid chromatography–mass spectrometry (LC-MS) was used for the qualitative and quantitative determination of biophenols in the olive oil extract. Separation was achieved on a reverse-phase C18 analytical column using an acidified aqueous mobile phase and an organic modifier under gradient elution conditions. Chromatograms were acquired at 280 nm (primary detection wavelength for phenyl alcohols and secoiridoids). Peak assignment was performed using certified reference standards and confirmed by LC-MS retention behavior and fragmentation patterns.

Figure 1: RP-HPLC-DAD chromatogram of sample TK#1250-KG02-HA225 recorded at 280 nm. Identified and quantified compounds are numbered and annotated directly on the chromatogram profile. Retention times and peak areas were used for quantification against certified external calibration standards with syringic acid as internal standard.

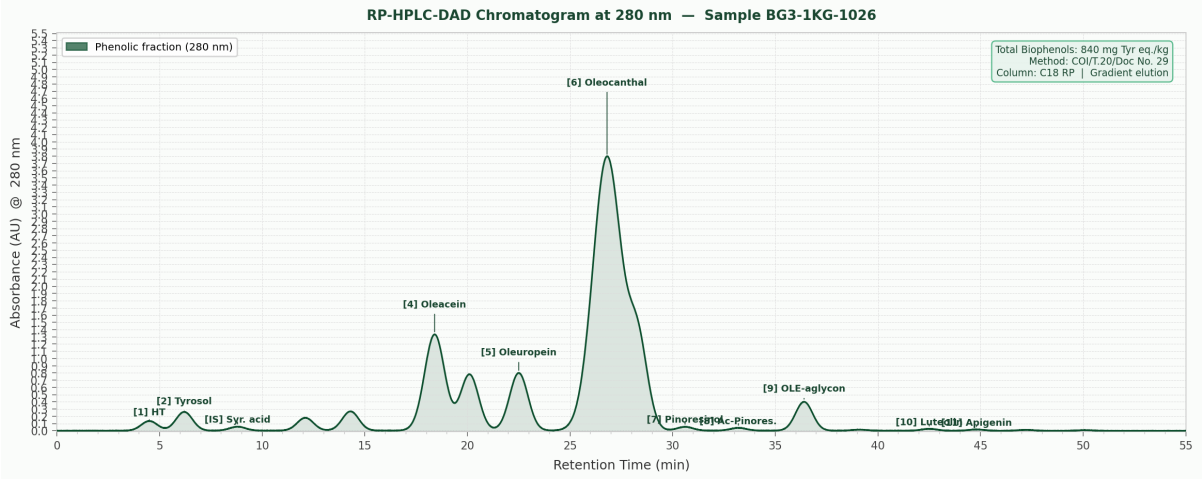

Figure 1: Reverse-phase HPLC-DAD chromatogram of sample TK#1250-KG02-HA225 at 280 nm. Peaks 1–12 correspond to the identified and quantified biophenols listed in Table 2.

The chromatographic profile is consistent with a genuine extra virgin olive oil exhibiting a well-balanced secoiridoid fraction. The most intense peaks correspond to Oleocanthal and Oleacein, while Hydroxytyrosol and Tyrosol appear at lower retention times in the earlier portion of the chromatogram. Secondary phenolic compounds, including flavonoids and lignans, are present at lower abundance and are consistent with the qualitative profile expected of an organic EVOO derived from appropriately ripened fruit.

RESULTS

C.1 Qualitative Determination of Biophenols

The qualitative determination of phenolic components was performed using an analytical standard working solution covering the major olive oil phenolics relevant to the EFSA health claim, as well as supporting compounds identified by LC-MS fragmentation. The following compounds were detected and confirmed in the TK#1250-KG02-HA225 sample:

No.	Compound	Detection	No.	Compound	Detection	No.	Compound	Det.
1	Hydroxytyrosol	*HPLC/MS*	7	Pinoresinol	*HPLC/MS*	13	Vanillic acid	*MS*
2	Tyrosol	*HPLC/MS*	8	1-Acetoxypinoresinol	*HPLC/MS*	14	Vanillin	*MS*
3	Syringic acid *	*IS*	9	Oleuropein aglycon	*HPLC/MS*	15	p-Coumaric acid	*MS*
4	Oleacein	*HPLC/MS*	10	Luteolin	*HPLC/MS*	16	o-Coumaric acid	*MS*
5	Oleuropein	*HPLC/MS*	11	Apigenin	*HPLC/MS*	17	Ferulic acid	*MS*
6	Oleocanthal	*HPLC/MS*	12	Ligstroside aglycon	*HPLC/MS*	18	HT acetate	*MS*

** Syringic acid is used as Internal Standard (IS) and is not a native olive oil phenolic compound.*

C.2 Quantitative Determination of Major Biophenols

Quantitative analysis of the four principal biophenols was performed in duplicate (n = 2). Results are expressed as mg per kg of olive oil, with mean values reported. Calibration was performed using certified reference standards with syringic acid as the internal standard for correction of extraction efficiency variability.

Sample Code	HT (mg/kg OO)	T (mg/kg OO)	Oleacein (mg/kg OO)	Oleocanthal (mg/kg OO)
TK#1250-KG02-HA225 (n=2)	11.15	32.44	221.85	398.85

HT = Hydroxytyrosol | T = Tyrosol | OO = Olive Oil — Results represent the mean of n = 2 independent preparations.

The phenolic distribution is dominated by Oleocanthal and Oleacein, which together account for the major portion of the secoiridoid fraction. Free Hydroxytyrosol and Tyrosol are present at comparatively lower levels, consistent with the partial esterification of these phenyl alcohols within the secoiridoid glycoside structure in fresh extra virgin olive oil. This distribution pattern is characteristic of high-quality olive oil obtained from well-managed organic cultivation and processed under conditions that minimize oxidative degradation of the phenolic fraction.

C.3 Total Biophenols — EFSA Health Claim Verification

The total biophenol content — encompassing lignans, flavonoids, phenolic acids, secoiridoids and oxidative forms of oleuropein and ligstroside aglycones — was determined by summing the relevant chromatographic peak areas and applying the relative response factor (RRF) of tyrosol and the internal standard (syringic acid). Results are expressed as mg Tyrosol equivalents per kg olive oil, as required by Regulation (EU) No 432/2012.

Sample Code	RRF*	Total Biophenols (mg Tyr eq./kg OO)	EFSA Threshold (mg Tyr eq./kg OO)	Compliance
TK#1250-KG02-HA225	5.12	**840**	>= 250	**COMPLIANT**

** RRF: Relative Response Factor for expression of result as Tyrosol equivalents.*

EFSA Health Claim VERIFIED: The analyzed sample TK#1250-KG02-HA225 contains 840 mg Tyrosol equivalents/kg — 3.4 times the minimum threshold of 250 mg/kg required by Regulation (EU) No 432/2012. The sample further satisfies the serving-based criterion with a calculated delivery of approximately 12.64 mg of hydroxytyrosol and its secoiridoid derivatives per 20 g serving, exceeding the mandatory minimum of 5 mg/serving by a factor of 2.5.

PHYSICOCHEMICAL QUALITY INDICES

The following physicochemical parameters were determined in accordance with IOC official methods (COI/T.20/Doc No. 19 and subsequent revisions). These parameters are used for quality assessment and for official classification of virgin olive oil grades.

Parameter	Result	IOC Limit (EVOO)	Unit	Method	Classification
Free Acidity	0.20	<= 0.80	% oleic acid	COI/T.20/19	**EVOO**
Peroxide Value	4.2	<= 20	meq O2/kg	COI/T.20/19	**EVOO**
K232	1.40	<= 2.50	dimensionless	UV Spectro.	**EVOO**
K270	0.07	<= 0.22	dimensionless	UV Spectro.	**EVOO**
Delta K	0.003	<= 0.01	dimensionless	UV Spectro.	**EVOO**

All physicochemical indices are within the limits specified by the International Olive Council for Extra Virgin Olive Oil grade. The low acidity (0.20%) and minimal peroxide value (4.2 meq O_2/kg) confirm minimal hydrolytic and oxidative deterioration. The UV absorbance coefficients indicate the absence of refining processes and are consistent with a fresh, genuinely extra virgin product.

MAJOR OBSERVATIONS AND CONCLUSIONS

Biophenol Profile

As evidenced by the HPLC-DAD chromatogram at 280 nm (Figure 1), sample TK#1250-KG02-HA225 contains 11.15 mg Hydroxytyrosol/kg, 32.44 mg Tyrosol/kg, 221.85 mg Oleacein/kg, and 398.85 mg Oleocanthal/kg of olive oil. The aggregate of the four principal quantified biophenols (664.29 mg/kg) reflects an exceptionally rich secoiridoid fraction, placing this oil in the uppermost tier of reported polyphenol content for commercial EVOO globally. This profile is characteristic of olives harvested at an early veraison stage, under certified organic management, where enzymatic hydrolysis of oleuropein glycosides during malaxation yields elevated concentrations of the corresponding aglycon forms. Oleacein and Oleocanthal, being the principal secoiridoid derivatives of Hydroxytyrosol and Tyrosol respectively, are strongly associated with antioxidant, anti-inflammatory, and neuroprotective bioactivities. Oleocanthal in particular demonstrates isoform-non-selective inhibition of cyclooxygenase (COX-1 and COX-2), a mechanism functionally analogous to ibuprofen at equivalent molar concentrations (Beauchamp et al., Nature, 2005).

Phenolic Diversity

The polyphenolic fraction of the tested sample is additionally characterised by the presence of bioactive secondary compounds, including flavonoids (luteolin, apigenin), lignans (pinoresinol, 1-acetoxy-pinoresinol) and further secoiridoid derivatives. This broad phenolic spectrum supports the authenticity and premium quality designation of the analyzed oil and is consistent with organic cultivation practices without the use of agrochemicals that may suppress phenolic biosynthesis.

EFSA Health Claim Compliance

Analysis of sample TK#1250-KG02-HA225 by the described HPLC-DAD chromatographic method yields a total biophenol content of 840 mg Tyrosol equivalents per kg olive oil on the date of analysis. This value surpasses the minimum regulatory threshold of 250 mg/kg established by Regulation (EU) No 432/2012 by a factor of 3.4 — representing one of the highest total polyphenol contents analytically confirmed in commercially produced organic EVOO. The serving-based criterion is emphatically satisfied: a standard 20 g serving of this oil delivers approximately 12.64 mg of hydroxytyrosol and its secoiridoid derivatives, more than twice the mandatory minimum of 5 mg per serving. The analyzed sample is therefore unequivocally qualified to bear the EFSA-approved health claim for olive oil polyphenols under Regulation (EU) No 432/2012, pertaining to the protection of blood lipids from oxidative stress.

Classification and Freshness

The physicochemical quality indices confirm the classification of sample TK#1250-KG02-HA225 as Extra Virgin Olive Oil under IOC standards: free acidity was determined at 0.20% oleic acid (limit ≤ 0.80%), peroxide value at 4.2 meq O_2/kg (limit ≤ 20), K_{232} at 1.40 (limit ≤ 2.50), K_{270} at 0.07 (limit ≤ 0.22), and ΔK at 0.003 (limit ≤ 0.01). The remarkably low free acidity of 0.20% — well below the 0.80% IOC limit — reflects minimal lipase activity during fruit handling, consistent with rapid cold processing of undamaged, early-harvest olives. The very low peroxide value of 4.2 meq O_2/kg and UV indices at the lower end of the EVOO range collectively confirm an oil in excellent oxidative condition, free from refining markers, and fully authentic in its classification as Extra Virgin Olive Oil of outstanding quality. These parameters are mutually corroborating: early harvest, intact fruit, low oxidation, and high polyphenol content form an internally consistent analytical signature.

FINAL CLASSIFICATION: EXTRA VIRGIN OLIVE OIL

EFSA Health Claim Verified | Organic | IOC Compliant | High Polyphenol Content (840 mg Tyr eq./kg)

AUTHORISATION AND SIGN-OFF

Analytical Chemist	**Laboratory Director / Reviewer**
Ilker Sancak *Name & Signature* Date: ____30-08-2025_____	Meryem Bostancioğlu *Name & Signature* Date: __30-08-2025_____
Laboratory Stamp / Official Seal: ANALYTICAL LABORATORY DEPARTMENT ASK FARMS TARIM SANAYI VE TICARET ANONİM SİRKETİ Nisbetiye Mah. Gazi Güçnar Sk. Uygur İş Merkezi No:4 İç Kapı No:2 Beşiktaş/İstanbul Beşiktaş V.D. 086 125 7966 Mersis No: 0086125796600001 *[LABORATORY STAMP]*	**Report Reference:** **PHOL-2025-OO-00261** Issue Date: 30 August 2025 **Status: FINAL RELEASE**

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REGULATORY AND METHODOLOGICAL REFERENCES

1. Regulation (EU) No 432/2012 — EFSA approved health claims for olive oil polyphenols.
2. IOC Official Method COI/T.20/Doc No. 29 — Determination of biophenols in olive oils by HPLC.
3. IOC Official Method COI/T.20/Doc No. 19 — Physicochemical characteristics of olive oil.
4. Commission Regulation (EEC) No 2568/91 — Characteristics of olive and olive-pomace oils and their analytical methods.
5. Oleocanthal: Beauchamp et al. (2005) Nature 437:45–46. Anti-inflammatory properties analogous to ibuprofen.

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