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A deep eutectic solvent-based one-step microextraction and hydrolysis of antioxidant precursors in extra virgin olive oil coupled to capillary electrophoresis analysis☆

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ABSTRACT

This paper proposes a one-step microextraction and hydrolysis approach for the capillary electrophoresis determination of the antioxidants hydroxytyrosol (HTy) and tyrosol (Ty) in extra virgin olive oil released from their antioxidant precursors. This method, based on the use of ethaline (a deep eutectic solvent obtained from choline chloride and ethylene glycol in a 1:2 M ratio) to which sulfuric acid was added at a 1 M concentration, allows the release of these phenolic compounds from their antioxidant precursors in which they are present as linked species in complex chemical structures.

The performance of the developed method was evaluated by initially using fortified seed oil samples and subsequently using samples of extra virgin olive oil (EVOO). Under the optimized conditions identified throughout our tests it was possible to obtain satisfactory results for the hydrolysis process conducted in ethaline with yields of approximately 94 ± 7% and detection limits for their electrophoresis determination, estimated for a signal to noise ratio of 3, equal to 40 μM for HTy and 60 μM for Ty. The results obtained by the proposed procedure were compared with those provided by an extraction and hydrolysis method followed by HTy and Ty chromatographic determination reported in the literature. The developed method allows the rapid and efficient extraction of antioxidants from oils using minimal amounts of reagents in significantly shorter times than conventional methods and, above all, avoiding any risk of solvent evaporation. It may therefore represent a valid alternative to the conventional methods currently used.

1. Introduction

In recent decades, analytical chemistry has undergone a strong transformation to respond to the need of environmental sustainability. This has led to a series of changes that can influence the various steps of the analysis process, from sample preparation to detection, and which are summarized in the 12 principles on which Green Analytical Chemistry (GAC) is based [1–3]. As a result, new extraction methods such as solid-phase and liquid-phase microextractions have emerged to significantly reduce both sample amounts and solvent volumes used in conventional extraction approaches, which are often time expensive, unsafe for operators and complex in terms of waste management [4–6]. In addition, microextraction approaches can be coupled to separation systems that operate at very low flow rates, such as Ultra High Performance Liquid Chromatography (UHPLC), or that are capable of operating with ultrasmall sample volumes (⟨10 nL) as Capillary Electrophoresis (CE) [7–9]. However, even the adoption of these techniques employing reduced amounts of solvents is not entirely decisive. In fact, in some production contexts, such as food industry, the use of even modest amounts of organic solvents entails a series of problems linked not only to their disposal as waste, but also to their storage.

To overcome these drawbacks, alternative solvents have been proposed, such as bioethanol, 2-methyltetrahydrofuran (2-MeTHF), 1,3-propandiol and terpenes (D-limonene, β-pinene, p-cymene) [10–12]. In this regard, deep eutectic solvents (DESs), which are low melting mixtures prepared by mixing two or more components that can form hydrogen bonds at different molar ratios and characterized by a low vapor pressure, non-flammability, a high thermal stability and a low

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thermal conductivity, can be considered an environmentally friendly alternative to conventional and nonconventional organic solvents [13–15].

The use of DESs has been proposed for several applications, such as solid phase microextractions (SPME), stir bar sorption extractions (SBSE), hollow fiber liquid phase microextractions (HF-LPME) and liquid phase microextractions (LPME) aimed at the recovery of phenol compounds, flavonoids, metal ions, and food contaminants [16–19]. The choice of the most suitable DES for efficient extraction depends on a number of properties, including melting point, density, viscosity, surface tension and polarity, which are in turn affected by the nature of the components present in the DES and their molar ratio. In particular, in the case of the use of DESs as extraction media for LPME, it is of fundamental importance that they display a low viscosity, since this maximizes both the rate and efficiency of the mass transfer, while simultaneously simplifying the management of the process when microsyringes are used, as very often happens in these extraction processes [20–23].

Furthermore, it is also possible to use DESs not only as extraction media, but also to carry out hydrolyses to break down complex compounds where the analytes are present in bound forms [24]. This is the case, for example, of hydroxytyrosol and tyrosol, which are the most important antioxidants frequently used to certify the nutritional quality of extra virgin olive oils (EVOO). They are often present as esters of glycosylated elenolic acids, in the form of secoridoids, such as oleuropein and ligstroside. Thanks to the relationship between the antioxidant activities and the concentration of these antioxidants, in 2012 the European Commission published a regulation (Reg. CE n°432/2012) [25,26] authorizing the use of a health claim for EVOO in relation to its polyphenol content. This claim reads "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" and is accompanied by the following condition of use "The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivative (e.g. oleuropein complex and tyrosol) per 10 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil".

Several methods are reported in the literature for the extraction of phenolic compounds from EVOO. The majority of these methods typically involve an extraction step, frequently conducted with a water-methanol solution (40:60 or 20:80 v/v), followed by hydrolysis in an acid environment, using sulfuric or hydrochloric acid at a high concentration (1 or 2 M) to break the ester bonds linking tyrosol and hydroxytyrosol in the complex compounds containing these phenolic moieties. The resulting extracts are then subjected to analysis by gas chromatography (GC) or high performance liquid chromatography (HPLC), usually adopting an UV/vis detector at a wavelength of 280 nm [27–31]. However, some methods avoiding the hydrolysis of the phenolic compounds of the original EVOO that rely on the sole extraction of polyphenols from olive oils have also been suggested [32,33].

The aim of this investigation was the development of a one-step innovative method of microextraction and hydrolysis with an acidified DES, coupled to a CE analysis for the determination of HTy and Ty in EVOO samples. This approach allows the use of a green extraction medium and of smaller volumes of sample. In particular, the DES ethaline was used, which is one of the most frequently studied DES, made of choline chloride and ethylene glycol in a 1:2 M ratio [34–39]. This extractant was used after addition of 1 M H_2SO_4, to make possible the simultaneous hydrolysis and extraction of the bound forms of HTy and Ty. To give acid properties to the DES used, we preferred to add sulfuric acid to ethaline, rather than replacing ethylene glycol with another hydrogen bonding donor (HBD), such as p-toluenesulfonic acid or trichloroacetic acid, in order to employ a DES with low viscosity [40–42]. The effectiveness of the adopted extraction medium to also carry out the hydrolysis of the esterified forms of HTy and Ty was verified by applying the method here proposed to oleuropein which was used as a prototype of esterified species. The hydrolysis and extraction procedure is followed by HTy and Ty analysis by capillary electrophoresis, a method particularly suited for analyzing small sample volumes, thus making it particularly suitable for the analysis of samples from microextractions. The performance of the analytical approach adopted is reported below, and the method was also applied to a real EVOO sample.

2. Materials and methods

2.1. Reagents and instrumentation

Aqueous solutions were prepared by deionized water obtained by a Purelab Flex 3 system (Elga, High Wycombe UK).

Hydroxytyrosol ($C_8H_{10}O_3$, ≥ 98.0%), tyrosol ($C_8H_{10}O_2$, 98.0%), hydroquinone ($C_6H_6O_2$, ≥ 99.0%), oleuropein ($C_{25}H_{32}O_{13}$, ≥ 98.0%), ethyl acetate ($C_4H_8O_2$, 99.9%), anhydrous ethylene glycol ($C_2H_6O_2$, 99.8%), choline chloride ($C_5H_{14}ONCl$, ≥ 98.0%), pentane (C_5H_{12}, ≥ 99.0%), sulfuric acid (H_2SO_4, 98.0%), methanol (CH_3OH, ≥ 98.0%), sodium hydroxide (NaOH, ≥ 98.0%), anhydrous sodium dihydrogen phosphate (NaH_2PO_4, > 99%), anhydrous sodium monohydrogen phosphate (Na_2HPO_4, > 99.0%) and sodium dodecyl sulfate ($NaC_{12}H_{25}SO_4$, ≥ 98.5%) were purchased from Sigma-Aldrich (Milano, I).

Extra virgin olive oil (EVOO) and sunflower oil samples were purchased from a local supermarket.

Phosphate buffers at a concentration of 50 mM (pH = 7) were prepared using the corresponding sodium salts mentioned above. Sodium dodecyl sulfate was added to these buffered solutions to achieve a 60 mM concentration when they were introduced into capillary electrophoresis adopting the micellar electrokinetic capillary chromatography (MECK) approach.

Sodium hydroxide solutions at two different concentrations of 0.1 M and 1 M, respectively, were used for the capillary conditioning and for the washing steps. In all preparations the reagents were weighed, brought to volume with deionized water and then the solutions were filtered by filters with pore size of 0.45 μm (Sartorius Stendim Biotech, Aubagne, F).

Standard solutions of tyrosol (Ty), hydroxytyrosol (HTy) and hydroquinone (H_2Q) were prepared in deionized water at a 10 mM concentration and then diluted with either aqueous sulfuric acid solution (0.1 M) or ethaline containing 0.1 M H_2SO_4 to obtain the desired concentrations (0.1; 0.3; 0.5; 1; 2 mM). Oleuropein solutions were prepared in pure ethaline or in ethaline containing H_2SO_4 (1 M) at a concentration of 5 mM.

Both fortified sunflower and EVOO samples (2 g) subjected to extraction and hydrolysis were preliminarily diluted with 5 mL of pentane to facilitate the subsequent handling of the extract and to ensure greater cleanliness of the oil introduced into the capillary during CE analyses. The fortified sunflower oil samples were analyzed after addition of 500 μL of an ethyl acetate solution containing 1.70 mg of H_2Q, 1.49 mg of HTy and 2.37 mg of Ty to 25 g of the original samples. Extra virgin olive oil (25 g) was fortified by adding 500 μL of ethyl acetate containing 1.76 mg of hydroquinone (H_2Q), this last as the internal standard.

All analyses were performed by a capillary electrophoresis (CE) system Agilent, model 7100 (Agilent Technologies, Santa Clara, USA) equipped with a diode array spectrophotometric detector (DAD) capable of operating in the UV/Vis range. Data acquisition and processing were carried out with a PC equipped with the Agilent CE software.

Untreated fused silica capillaries were cut from a hank purchased from Polymicro Technologies (Phoenix, USA). The internal and external diameters of the capillaries were 50 and 360 μm, respectively, while their total length was 48.5 cm, with a real effective length of 40 cm.

The UV detection was carried out at a wavelength of 280 nm, in agreement with the literature referring to the separation of HTy and Ty by HPLC [25,27,30,31,43,44].

An ultrasonic bath Bransonic 1210 ultrasonic cleaner purchased

from Branson (Danbury, USA), a Vortex equipment (International PBI SPA, Milano, I) and a centrifuge (Tecnopound s.r.l., Ravenna, I) were used to perform microextractions.

A concentrator (Techne Sample concentrator, Bibby Scientific, Stone, Staffordshire, UK) was used to heat the samples in order to facilitate acid hydrolysis and to concentrate the sample after extraction and hydrolysis in the case of using, for the sake of comparison, the conventional procedure reported in the literature [25,27,30,31,43]. Absorbance measurements were performed with a commercial benchtop spectrophotometer Varian Cary 50 bio (Victoria, AUS).

2.2. Ethaline preparation

The hydrophilic deep eutectic solvent (DES) ethaline was prepared by mixing choline chloride (ChCl) and ethylene glycol (EG) in a 1:2 M ratio. With this purpose, choline chloride (ChCl) were added to ethylene glycol and the obtained blend was mixed and heated at 80 °C for about 2 h, until a clear solution was obtained [34,45].

2.3. Extraction of HTy and Ty from EVOO after hydrolysis

The microextraction and subsequent hydrolysis with ethaline was conducted by introducing 2 g of oil sample in a centrifuge tube, where 5 mL of pentane and 100 μL of ethaline containing 1 M of sulfuric acid were added. The mixture thus obtained was vortexed for 2 min, sonicated at room temperature for 15 min and then centrifuged at 4000 rpm for 10 min. Subsequently, the DES phase (present as a drop at the bottom of the centrifuge tube) was picked up with a microsyringe and transferred to a 2 mL glass vial where the hydrolysis phase, aimed at releasing HTy and Ty, was carried out by heating at 80 °C for 2 h. The procedure was then completed diluting 10:1 *v*/v the hydrolyzed extract by adding deionized water to acquire a H_2SO_4 concentration 0.1 M compatible with our CE measurements. From this solution, 100 μL of extract were taken and inserted into a vial for the subsequent CE analysis of the sample thus obtained.

The results found with this procedure were compared with those obtained by adopting an alternative conventional method described in the literature [25,27,30,31,43]. This method, slightly adapted to CE analyses, is based on the use of a CH_3OH/H_2O solution 80:20 *v*/v as the extraction medium. More specifically, 2 g of oil sample were weighed into a centrifuge tube where 5 mL of CH_3OH/H_2O (80:20 *v*/v) solution were then added. The obtained mixture was vortexed for 1 min, sonicated at room temperature for 15 min and then centrifuged at 5000 rpm for 25 min. Subsequently, the hydroalcoholic phase was transferred into a 5 mL cylindrical container where it was concentrated to about 300 μL by using a nitrogen flow. This concentrated sample was transferred to a 2 mL vial where it was brought to the exact volume of 300 μL with deionized water and subsequently mixed with 300 μL of a 1 M sulfuric acid aqueous solution to achieve hydrolysis by heating at 80 °C for 2 h. Subsequently, 2.4 mL of deionized water were added to this hydrolyzed extract to bring the final concentration of sulfuric acid in the solution to 0.1 M. Finally, 100 μL of this solution were taken and placed in a CE vial for their CE analysis.

2.4. Electrophoretic conditions for the separation of HTy and Ty

To activate the inner surface and to generate SiO^- ions from silanol groups (-SiOH), each new capillary was conditioned using both two NaOH solutions with concentrations of 1 M and 0.1 M, respectively, and deionized H_2O. This conditioning was performed flushing the capillary, at a pressure of 950 mbar, with the mentioned three aqueous media in the following order: NaOH solution at a concentration of 1 M (30 min), NaOH solution at a concentration of 0.1 M (30 min) and deionized H_2O (15 min). In order to reactivate the capillary before performing each set of analyses, it was rinsed with 1 M NaOH solution (3 min), 0.1 M NaOH solution (3 min) and with the electrophoretic medium (3 min). The chosen electrophoretic medium used as the running buffer was a 50 mM solution of phosphate buffer at pH 7, prepared by sodium monohydrogen phosphate and sodium dihydrogen phosphate, at which sodium dodecyl sulphate (SDS) at a 60 mM concentration was added in order to profit of the MECK approach. The electrophoretic separation was carried out applying a potential of 25 kV for 10 min. During the electrophoretic analysis the temperature of the capillary was kept constant at 25 °C. Samples were injected by a hydrodynamic injector (50 mbar for 3 s).

3. Results and discussion

3.1. Conditions adopted for CE analysis of HTy and Ty

As reported in the Introduction section, the aim of this research was to test the feasibility of the microextraction and hydrolysis of antioxidant precursors present in extra virgin olive oils using very small amounts of both the sample and the DES ethaline as extractant. Therefore, it was necessary to adopt an analytical method suited to detect the small amounts of HTy and Ty released in this procedure. To this end, it was deemed appropriate to use capillary electrophoresis, which is a method particularly suited to the analysis of small sample volumes.

The electrophoresis procedure was optimized by conducting several tests on HTy and Ty aqueous synthetic samples and by using the following different aqueous separation media: $H_3PO_4/H_2PO_4^-$ buffer at pH = 2.5, $H_3PO_4/H_2PO_4^-$ buffer at pH = 4.5, $H_2PO_4^-/HPO_4^{2-}$ buffer at pH = 7 and HPO_4^{2-}/PO_4^{3-} buffer at pH = 10.5. It was thus possible to verify that the best performance could be obtained by using a 48.5 cm long capillary (effective length 40 cm), using a 50 mM phosphate running buffer (pH = 7) containing 60 mM of SDS and applying a potential of 25 kV. These separation conditions ensured good resolution, short analysis times and high sensitivity by using UV detection of HTy and Ty at a wavelength of 280 nm.

A typical electropherogram recorded under these optimized conditions is reported in Fig. 1 which shows that a good separation can be achieved for 1 mM HTy and Ty solutions.

The electrophoretic conditions thus defined were then adopted to

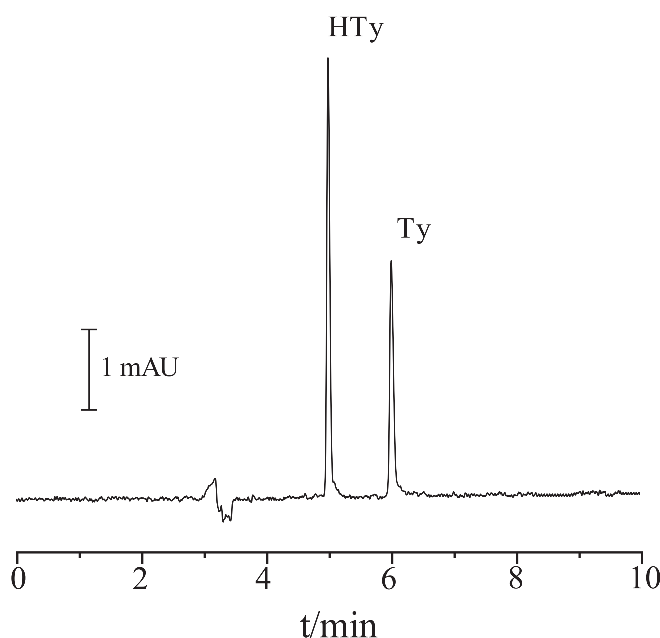

Fig. 1. Electropherogram recorded in an aqueous solution containing 1 mM HTy and Ty. CE conditions: 50 mM phosphate buffer (pH 7) + 60 mM SDS as the running buffer. Injection: hydrodynamic (50 mbar × 3 s). Applied potential: 25 kV (25 °C).

analyze HTy and Ty synthetic samples prepared by using increasing concentrations of these antioxidants The recorded peaks displayed an area which exhibited a linear trend in the concentration range 0.1–2.0 mM, thus allowing the construction of the corresponding calibration plots. Based on peak areas, the following calibration plots were found: $y = 18.674C_{HTy} + 0.140$ ($R^2 = 0.993$) and $y = 13.116C_{Ty} + 0.040$ ($R^2 = 0.998$) for HTy and Ty, respectively, where y is the peak area in mAU s. The corresponding detection limits (LODs), estimated for a signal to noise ratio of 3, turned out to be 0.04 mM and 0.06 mM for HTy and Ty, respectively. Relative standard deviations (RSD), calculated from the peak area values recorded in different tests ($n = 10$) of 5.6% and 2.6% for HTy and Ty, respectively, were found. The achieved results demonstrated that the CE detection was characterized by a good repeatability and sensitivity.

These CE conditions, only slightly modified as described below, were then also adopted in the analysis of the oil samples resulting from extraction and hydrolysis.

3.2. Verification of the possibility of carrying out the hydrolysis in ethaline

To verify whether ethaline is a suitable medium to conduct the hydrolysis step, some preliminary tests were conducted using oleuropein, whose structure includes the hydroxytyrosol linked to the elenolic acid via an ester bond [46] and which is present in EVOO, even though in trace amounts. This compound should be considered an excellent model for antioxidant precursors because in all these species, HTy and Ty are always present in the esterified form. Therefore, we used oleuropein as a reference species for phenolic antioxidants.

The hydrolysis was conducted on 100 μL of ethaline solutions containing 1 M of sulfuric acid and 5 mM oleuropein. As verified by us through several tests conducted by using different amounts of ethaline, this DES amount was sufficient to make the handling and sampling of the extraction phase sufficiently easy. At the same time, this volume proved to be sufficient to dissolve all phenolic compounds potentially present in the sample, while ensuring a good level of concentration of the extracted analytes. We verified this circumstance by adding increasing amounts of HTy to the aforementioned volume of ethaline containing 1 M H_2SO_4.

By carrying out a series of tests during which both different temperatures (50–90 °C) at which the hydrolysis was carried out and different durations (1 - 3 h) were assayed, it was possible to verify, by CE analysis conducted by resorting to the partially modified conditions described below, that the maximum HTy yields (94 ± 7%) were obtained by heating the extract at 80 °C for 2 h.

Under these conditions, it is expected that the hydrolysis process can be carried out profitably, allowing the breaking of the glycosidic and ester bonds and, consequently, the almost complete release of hydroxytyrosol, as also occurs in aqueous media.

Oleuropein solutions, both before and after the hydrolysis process at 80 °C for 2 h, were subjected to spectrophotometric analysis after being diluted with water (1:20 v/v) to acquire a concentration compatible with spectrophotometric measurements. The obtained spectra are compared to each other in Fig. 2, which shows a close similarity between them since in both an absorption band at 280 nm is displayed. This band is in fact due to the dihydroxyphenyl group which is present in both oleuropein and hydroxytyrosol released by hydrolysis. Of course, this spectrophotometric analysis did not allow the evaluation of the occurrence of the hydrolysis process, but it allowed us to verify that during the heating process at 80 °C for 2 h no interfering species was formed due to the use of the DES or its possible degradation caused by the presence of a strong acid (H_2SO_4) at a rather high concentration.

Confirmation of the progress of the hydrolysis process was instead obtained by comparing the results obtained in the CE analyses of the oleuropein samples before and after their heating at 80 °C for 2 h.

It is important to underline, however, that the CE analyses conducted on these ethaline-containing samples required a partial modification of

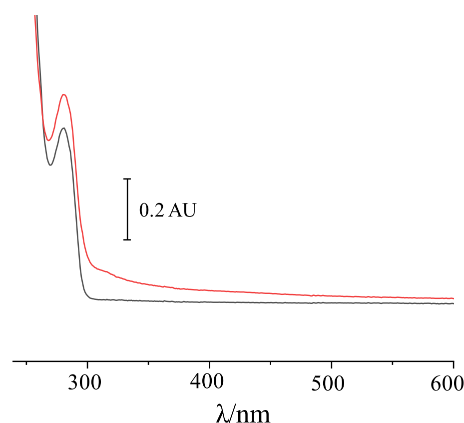

Fig. 2. Comparison of UV–vis spectra recorded for a 5 mM oleuropein solutions in ethaline containing 1 M H_2SO_4 before (black line) and after (red line) the hydrolysis process conducted by heating these samples at 80 °C for 2 h. In both cases, these spectra were recorded after dilution with water (1, : 20 v/v) to acquire a concentration compatible with spectrophotometric measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the method described in the previous Section, since the direct injection of the ethaline-hydrolyzed samples into the electrophoresis system completely compromised the analysis results. In fact, due to the high ionic strength of this DES, significantly higher than that of the electrophoretic medium, immediately caused a sharp increase in the background current as soon as these ethaline-containing samples entered the capillary. To overcome this problem, several tests were carried out using 1 mM solutions of HTy and Ty in ethaline containing 1 M H_2SO_4 diluted with water in DES/H_2O ratios of 50:50, 30:70 and 10:90 v/v, respectively. The results of these tests showed that the best performance in terms of noise and peak shape could be achieved by diluting the acid extract with water in a ratio of 10:90 v/v.

By adopting these partially modified conditions for the analysis of freshly prepared oleuropein solutions, no CE peak was observed even when the analysis time was extended to 30 min, as shown in Fig. 3 (black line), thus confirming that the addition of ethaline to the oleuropein

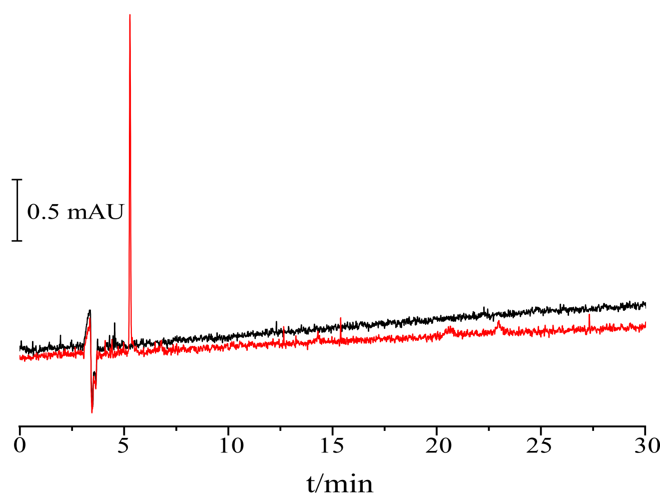

Fig. 3. Electropherogram recorded for a 5 mM oleuropein solutions in ethaline containing 1 M H_2SO_4 before (black line) and after (red line) the hydrolysis process conducted by heating these samples at 80 °C for 2 h. CE conditions: 50 mM phosphate buffer (pH = 7) + 60 mM SDS as the running buffer. Injection: hydrodynamic (50 mbar × 3 s). Applied potential: 25 kV at 25 °C. In both cases, these CE runs were recorded after dilution with water (1090 v/v). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solution was unable to lead to the formation of either HTy or other degradation compounds. Conversely, the analysis of solutions containing the same amount of oleuropein after being subjected to the hydrolysis process, led to the appearance of a peak at 5.3 min due to hydroxytyrosol (see the red line in Fig. 3), as we verified by adding controlled amounts of HTy to these solutions.

Comparison of Fig. 3 with Fig. 1 highlights that these partially modified CE analysis conditions led to a slightly higher retention time for HTy. A slightly higher retention time was also observed for Ty when these modified analysis conditions (ethaline containing 1 M H_2SO_4 diluted with water at a DES/H_2O ratio of 10:90 v/v), were adopted for analyzing specially prepared synthetic tyrosol and hydroxityrosol samples.

Conversely, no variation was observed for the peak areas for these two species due to the presence of ethaline, thus making it possible the use of the corresponding calibration plots reported in Section 3.1 constructed in the absence of this DES. This was also verified by purposely introducing increasing concentrations of HTy and Ty into the above-mentioned medium.

3.3. Evaluation of the recovery of the ethaline-based microextraction

The use of ethaline to carry out the microextraction process proposed here was suggested by information on the properties of this DES that were recently become available. Recent investigations reporting that DESs based on ethylene glycol (such as ethaline) or glycerol (such as glyceline) show a higher enrichment factor than pure ethylene glycol and glycerol in the microextraction of phenolic compounds such as caffeic, ferulic and cinnamic acid from vegetable oil samples [16]. This was attributed to the higher hydrogen bonding ability and electrostatic interactions of these DESs with the target analytes compared to those of pure ethylene glycol and/or glycerol [16]. Moreover, the extraction efficiency of ethaline was found to be higher than that of glyceline [47], reasonably because the viscosity of glyceline (259 cp at 25 °C [16]) is rather higher than that of ethaline (36 cp at 25 °C [16]). Furthermore, phenolic compounds can be considered a type of HBD such as ethylene glycol and glycerol and can thus interact with the halide anion of choline chloride [48]. In addition, DES components can react with the target compound or sample matrix, so that DES does not only serve as a solvent in the extraction process [49–51].

To evaluate the relative recovery (RR%), seed oil samples were preliminarily used because in these oils HTy and Ty, as well as the corresponding promoters, are totally absent, as also verified by us. These samples were spiked with controlled amounts of both HTy and Ty, as well as of H_2Q which was used as an internal standard to overcome any possible problem and error that may occur during extraction, injection or analysis. Hydroquinone (H_2Q) was chosen as the internal standard, since its phenolic nature guarantees similar behavior to that of HTy and Ty during extraction in terms of interactions with ethaline. Furthermore, this compound is expected to be characterized by a retention time different from those of HTy and Ty and therefore did not interfere with their quantification.

Based on peak areas recorded by preliminary CE analysis on synthetic samples prepared by using increasing concentrations of H_2Q in ethaline containing 1 M H_2SO_4 and diluted with water (10:90 v/v), the calibration plot also for this internal standard could be constructed, whose equation was $y = 14.551C_{H2Q} + 0.053$ ($R^2 = 0.999$).

The amounts of HTy, Ty and H_2Q added are reported in Table 1, together with the relative recoveries which were calculated with the following equation:

$$RR\% = 100\ (C_{found} - C_{real})/C_{added}$$

where C_{found} is the concentration determined after the extraction, C_{added} is the concentration achieved following the addition of each compound and C_{real} is the original concentration of the analyte in the sample, which was not considered since the original concentration of HTy, Ty and H_2Q in our seed oil samples was equal to 0 [52,53].

Table 1
Analytical performance of the DES-based microextraction found from the analysis of a fortified sunflower oil sample.

Analyte	Initial amount (mg/kg)	Added amount (mg/kg)	Average amount found (mg/kg)	Average relative recovery % (*n* = 5)	RSD % (n = 5)
H_2Q	0	66.64	56.18	84.3	6.8
HTy	0	58.41	47.49	81.3	6.3
Ty	0	92.91	79.51	85.6	5.6

Although in this case hydrolysis was not necessary, since the added compounds were already in free form, the procedure involving the sample heating at 80 °C for 2 h was still used to check whether this procedure led to losses of analytes. Therefore, 100 μL of ethaline containing 1 M H_2SO_4 were added to the fortified seed oil samples, after their weighing. They were then vortexed for 2 min, sonicated for 15 min and centrifuged at 4000 rpm for 10 min. The sedimented layer obtained, corresponding to 100 μL of acid ethaline (for the presence of 1 M H_2SO_4), was picked up with a microsyringe and heated at 80 °C for 2 h to simulate the hydrolysis process. As it is well known, the use of ultrasound accelerates the mass transfer of the analytes by increasing the interface between the two immiscible phases, thus reducing the time required to achieve equilibrium conditions and increasing the preconcentration factor. As it can be seen from the electropherogram shown in Fig. 4 the three peaks of H_2Q, HTy and Ty appeared sharp and well defined and no further interfering peak was observed.

3.4. Application of the overall procedure to EVOO samples

Some samples ($n = 7$) of extra virgin olive oil purchased at a local supermarket (2 g) were preliminarily diluted with pentane (5 mL) which was used instead of the more widely adopted hexane because it is less toxic [54].

EVOO samples were then subjected to the DES-based microextraction and hydrolysis procedure here proposed and then analyzed by CE after being fortified with H_2Q which was used also in this case as internal standard. Moreover, to further evaluate the possible occurrence of interference caused by the presence of the internal standard, these tests were performed not only on the H_2Q-fortified samples, but also on

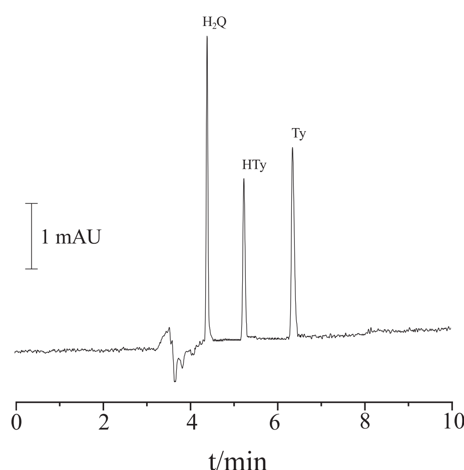

Fig. 4. Electropherogram recorded for a sunflower oil sample fortified with the H_2Q, HTy and Ty amounts reported in Table 1, after the simulation of the hydrolysis process consisting in the heating at 80 °C for 2 h. CE conditions: 50 mM phosphate buffer (pH 7) + 60 mM SDS as the running buffer. Injection: hydrodynamic (50 mbar × 3 s). Applied potential: 25 kV (25 °C).

the as-is samples. CE analyses of these not-fortified samples showed perfectly coincident profiles with those displayed by fortified samples, except for the absence of the H_2Q peak for samples where this internal standard had not been added, thus highlighting that the addition of the internal standard did not cause any impact or interference.

The typical electropherogram recorded for these samples added with H_2Q is shown in Fig. 5. The corresponding peak areas were converted to the corresponding concentrations using the calibration plots previously constructed for H_2Q, HTy, and Ty. The values thus obtained are listed in Table 2, where they are compared with those acquired using the method described in the literature and mentioned above [27,30,31].

This comparison highlights that the method proposed by us allows slightly lower recoveries than those obtainable using a hydroalcoholic solution as extracting. On the other hand, this was expected since extraction with a hydroalcoholic solution involves the use of larger volumes of extractant. In all cases, these values were satisfactory considering the range 80–110% proposed by AOAC [55].

However, the extraction method we propose allows us to obtain a more than satisfactory extraction yield, respecting the principles of GAC [56], which are instead not respected by extraction methods with hydroalcoholic solutions. Furthermore, the ethaline extraction method involves the use of small amounts of chemicals and allows the extraction to be carried out in significantly shorter times, since it does not require any concentration step.

4. Concluding remarks

The microextraction and hydrolysis method conducted in ethaline proposed here allows the release and extraction of HTy and Ty from extra virgin olive oils with minimal amounts of reagents (100 μL of DES), thus making it highly environmentally friendly. Even the operational simplicity and the short extraction time, significantly reduced compared to that required by conventional methods, thanks to the elimination of the pre-concentration step, make the microextraction and hydrolysis method based on DES an approach undoubtedly in line with the principles of green and white analytical chemistry [3]. In fact, good analytical performance is effectively attained through the use of reagents and instruments that offer benefits in both environmental sustainability and cost-efficiency. Furthermore, the microextraction method with ethaline not only allows a simpler and faster extraction than conventional methods, but it facilitates the scission of ester bonds and the release of HTy and Ty from the compounds in which these antioxidants are present in esterified forms as constituents of complex molecules, thanks to the acid environment in which it is carried out.

Furthermore, this extraction procedure here proposed is the most suitable to be coupled with analyses by capillary electrophoresis which is an instrumental analytical technique characterized by high separation efficiency and excellent sensitivity and which allows rapid analyses using an aqueous separation medium and very small volumes of samples. The use of low-cost fused silica capillaries, which can be easily replaced, and the absence of a stationary phase, also serve to eliminate a multitude of critical issues associated with the cleaning of the injected sample, thus simplifying the microextraction procedure. It is also noteworthy that the CE technique lends itself to miniaturization, facilitating the development of portable and cost-effective instruments that are optimally suited for microextraction procedures that relies on the use of small volumes of both sample and extraction media.

In addition to being economically advantageous, this approach allows a greater number of samples to be analyzed daily with a lower environmental impact. The unique characteristics of the extraction method and separation technique adopted make this approach particularly suitable for environments where solvent storage and chemical waste management can present challenges, such as food production facilities.

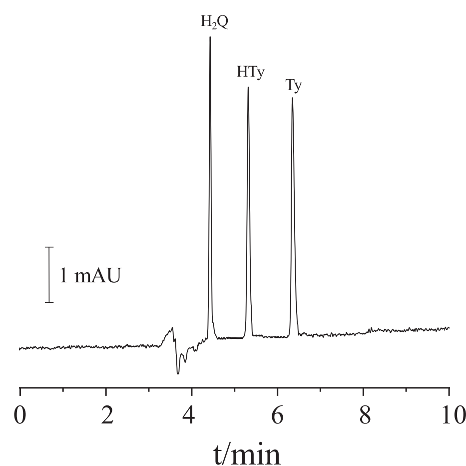

Fig. 5. Electropherogram recorded for an EVOO sample added with H_2Q as internal standard after the hydrolysis process consisting in the heating at 80 °C for 2 h. CE conditions: 50 mM phosphate buffer (pH 7) + 60 mM of SDS as the running buffer. Injection: hydrodynamic (50 mbar × 3 s). Applied potential: 25 kV (25 °C).

Table 2
Analytical performance of the DES-based microextraction found for the analysis of an EVOO sample.

Analyte	Amount found with the microextraction and hydrolysis method here proposed (mg/kg)	RSD % (n = 5)	Amount found with the microextraction and hydrolysis method from literature [27,30,31] (mg/kg)	RSD % (n = 5)
H_2Q	59.84	6.8	69.74	6.3
HTy	66.79	6.3	81.42	7.1
Ty	102.28	5.6	120.61	6.6

CRediT authorship contribution statement

Michele Abate: Methodology, Investigation, Formal analysis, Data curation. **Eleonora Dametto:** Methodology, Investigation, Formal analysis, Data curation. **Gino Bontempelli:** Writing – review & editing, Formal analysis. **Nicolò Dossi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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