Food Analytical Methods
https://doi.org/10.1007/s12161-019-01492-w

Lab-On-A-Chip Extraction of Phenolic Compounds from Extra Virgin Olive Oil

Kenia Chávez Ramos[1] · Luis Fernando Olguín Contreras[2] · María del Pilar Cañizares Macías[1]

Received: 2 January 2019 / Accepted: 21 March 2019

Abstract

A novel liquid–liquid extraction method of phenolic compounds from extra virgin olive using microfluidic technique was developed. The microdevice of polydimethylsiloxane (PDMS) was designed in two parts; the first one to carry out the extraction of polyphenols using only an alkaline aqueous solution and the second one to develop the reaction product using Folin–Ciocalteu reagent, which was measured at 730 nm at the microchip exit. Hydrodynamic and chemical parameters, such as flowrates of extraction and reaction, length of microchannels, extraction pH, extraction buffer concentration, and concentration of Folin–Ciocalteu reagent, were evaluated. Although all parameters were important, the results showed that pH, carbonate buffer concentration, and the Folin–Ciocalteu reagent concentration were significant factors and that the increase in the length of the extraction coil enhanced the extraction percentage. The results showed higher extraction efficiency by the microfluidic method, between 46 and 67%, than for the other two batch extraction methods.

Keywords Polyphenols · Lab-on-a-chip · Folin–Ciocalteu assay · Liquid–liquid microfluidic extraction · Extra virgin olive oil

Introduction

Extra virgin olive oil (EVOO), obtained by cold-pressed extraction of olive fruits, is an important constituent. The chemical composition of EVOO is complex and includes a heterogeneous mixture of compounds, among which, phenolic compounds, named as well as polyphenols, are distinguished. Polyphenols include different substances, like phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavonoids. Phenolic compounds have been studied for being an excellent source of antioxidants, and its properties cause benefits on human health as prevention of chronic diseases (Tripoli et al. 2005). There are several available methods to extract the phenolic fraction from food samples, among these, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are the most used (Bendini et al. 2003; García-Salas et al. 2010).

In LLE, a mixture of two immiscible solvents is selected to guide the separation process through the distribution of the analyte between the two phases. In the particular case of olive oil's polyphenol extraction, solvents, like methanol or ethanol diluted in water and n-hexane, are recommended (Bonoli et al. 2003; International Olive Council 2009; Pirisi et al. 2000). However, this technique requires expensive and hazardous organic solvents and a long time per analysis, which can affect the stability of the phenolic compounds and their final total concentration. In the recent years, the search of new extraction methods has become increasingly important and the draw on liquid-phase microextraction (LPME) and microtechnology has taken place (Monasterio et al. 2013; Spietelun et al. 2014). Thus, the use of microfluidic devices has shown a rapid growth with the development of the so-called "laboratory on a microchip" (lab-on-a-chip), which is a device that has integrated laboratory functions, such as pretreatment of samples, mixing of reagents, and separation of products within channels whose size is between tens and hundreds of microns (Lim et al. 2010; Ríos et al. 2012; Temiz et al. 2015).

There are different ways to manufacture a microdevice; photolithography and soft lithography are widely used to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12161-019-01492-w) contains supplementary material, which is available to authorized users.

✉ María del Pilar Cañizares Macías
pilarm@unam.mx

[1] Departamento de Química Analítica, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México D.F., Mexico

[2] Laboratorio de Biofisicoquímica, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México D.F., Mexico

Published online: 08 April 2019

fabricate the mold and polydimethylsiloxane (PDMS) microfluidics, being relatively easy and economical techniques (Zaouk et al. 2006a, b). The application of microfluidic devices is wide and diverse in different scientific areas. Many works, referent to liquid–liquid microextraction, have been developed and reported in the last years, being the microdroplet formation one of the extraction techniques most employed (Kralj et al. 2007; Maruyama et al. 2004; Mary et al. 2008; Salik et al. 2011).

Once extracted, phenolic compounds can be quantified through the Folin–Ciocalteu (F–C) assay as total polyphenol content (TPC). The F–C reaction is complex, but it is known that during the process, a transfer of electron reaction between phenolate ions and molybdates/tungstates occurs at basic pH value. The resulting blue-colored product is spectrophotometrically detected at 730 nm (Sánchez-Rangel et al. 2013). The F–C method is one of the most used for spectrophotometric quantification of total polyphenols. Although it has reactions with other types of reducing agents, this method is used in the food industry to give a close ratio of the total amount of polyphenols. Since each polyphenol has different reducing force against the F–C reagent, the reduced complex concentration will depend on the concentration of the major polyphenol; thus, the total concentration of polyphenols was referenced against an established standard, such as gallic acid or tannic acid.

The TPC in extra virgin olive oil is generally an interval from 200 to 1500 mg equivalents of a standard/kg of olive oil, depending on different factors which are involved in its final content, such as maturation of the olive, extraction system, kind of olive, and climatic and agronomic conditions of the olive cultivation (Lozano et al. 2009). At present, the technique of HPLC coupled to UV and/or mass spectrometry has been used to identify a large part of polyphenols in olive oil, but, due to the lack of commercial standards, it is not possible to quantify each one of them. This is why the quantification of total polyphenols by the F–C method is an indispensable parameter in the quality control of antioxidants in this type of samples.

In this work, a novel liquid–liquid extraction method to extract polyphenols from extra virgin olive oil and derivatization reaction with Folin–Ciocalteu reagent using microfluidic technique was developed. The microdevice was designed in two parts; the first one to carry out the extraction of polyphenols using only an alkaline aqueous solution and the second one to develop the reaction product using the Folin–Ciocalteu reagent. Different hydrodynamic and chemical parameters, such as flowrate of extraction and reaction, length of microchannels, extraction pH, extraction buffer concentration, and concentration of Folin–Ciocalteu reagent, were evaluated, being all them parameters important in the proposed extraction and reaction procedures by microfluidic.

Methodology

Instruments

Spin coater (Laurell Technologies), a plasma generator (Electro-Technic Products), a UV lamp (Black Ray B-100AP, UVP), a digital hotplate (CIMAREC), and a heating oven (Heating Incubator Prolab) were used to carry out the lithography and photolithography processes. For the characterization of the microchips, a Leica 20× optical microscope and a Phantom camera coupled to the microscope were used.

Syringe pumps (New Era Pump Systems), 5 mL glass syringes (Hamilton), 0.022 in ID Teflon tubing, optical fibers of 400 μm, a 1-cm optical flow Z cell, a UV–Vis-NIR light source, and a UV–Vis USB4000 detector from Ocean Optics were used for the liquid–liquid extraction of polyphenols from olive oil and its quantification in the microchips. For the quantification of polyphenols using the batch methods, a Varian UV–Vis Cary I detector and a 1-cm optical path quartz cell were used.

Materials

Silicon wafers of 7.5 cm in diameter and 381 ± 25 μm in depth were purchased from Silicon Valley Microelectronics, USA; SU-8 3025 photoresist epoxy resin and SU-8 Developer were purchased from MicroChem Inc., USA; and polydimethylsiloxane (Sylgard 184) was purchased from Dow Corning Corporation.

Reagents for quantification were Folin–Ciocalteu reagent (Merck), sodium hydroxide (Fermont), sodium bicarbonate (Baker), n-hexane (Baker), and tannic acid (Sigma-Aldrich), which were used as standard. Methanol, 2,4,6-trichlorophenol, and 2,4-dichlorophenol (Sigma-Aldrich) were used to evaluate the extraction efficiency of the proposed microchip and the batch methods.

Extra Virgin Olive Oil Samples

Six EVOO samples (*Sierra de Gata*, Spain; *Casas Hualdo*, *Cornicabra*, Spain; *Las doscientas Blend*, *Arbequina/Picual*, Chile; *Las doscientas*, *Picual*, Chile; *Pons tradicional*, *Frutado*, Spain; *Pons ecológico*, *Natural*, Spain) were used to evaluate the proposed method, whose results were compared with those obtained by two batch extraction procedures.

Microdevice Manufacture

Microchannel Design

Six different prototypes of microdevice were drawn employing the Adobe Illustrator CC 2014 software within a 70-mm circumference and printed on acetate negative to get

the optical mask and construct the microdevices by photolithography and soft lithography. In Fig. 1 are shown the designs evaluated. Different lengths and width of the microchannels of reaction and extraction were evaluated in order to select the one that would provide the highest efficiency.

For design 6, only one reactor was used with the aim of that the extraction and reaction of polyphenols were at the same time. For the other designs (from 1 to 5), two parts were evaluated; in the first part, only extraction was carried out introducing the alkaline solution and the sample through inputs 1 and 2 and, in the second part, the reaction with Folin–Ciocalteu reagent, introduced through input 3, occurred at the same time as the extraction continued.

Photolithography Process

A silicon wafer was put in the spin coater, and a program of 500 rpm for 10 s with acceleration of 100 rpm/s continued at 2000 rpm for 30 s with acceleration of 300 rpm/s was followed. During the first 5 s, 2-propanol was added with the purpose of cleaning the silicon wafer. Immediately, it was put on a hot plate at 200 °C for 10 min and then let to cool at room temperature. One more time, the silicon wafer was put in the spin coater, and 3 mL of SU-8 3035 photoresin was aggregated, applying the program described previously with the purpose of creating a layer of 50 μm; immediately, the soft bake was carried on heating at 105 °C for 15 min (this step was performed twice to get a final layer around 100 μm).

The exposure step was performed using a UV lamp, putting the negative mask of the designs on the silicon wafer between the two glasses. After that, the post-exposure bake took place at 75 °C for 1 min and at 105 °C for 5 min.

As final step, the silicon wafer was dived in a vessel containing propylene glycol monomethyl ether acetate (Sigma-Aldrich) eliminating the no-polymerized photoresin, washed with 2-propanol, and put inside a Petri dish (MicroChem (n.d)).

Soft Lithography Process

A Sylgard 184 silicone elastomer kit was used to fabricate PDMS replicas. The base monomer and curing agent, mixed in a ratio of 10:0.9 *w/w*, were spilled into the Petri dish removing all the air bubbles in vacuum. The mold was heated at 70 °C for 24 h, to finish the polymerization. The resulting microdevices were cut with a scalpel, drilling the inlets and outlets of the microchannels with a perforator of 0.75 mm in diameter, and glued on glass-employing plasma discharge. The final microchips were left to rest for 48 h before use.

Microchip Characterization

The negative acetate masks were observed under a microscope (Steindorff) and compared with PDMS microchips once fabricated to discard the possible irregularities in transfer.

The characterization of the prototypes manufactured was carried out using a microscopic reticle picture and a

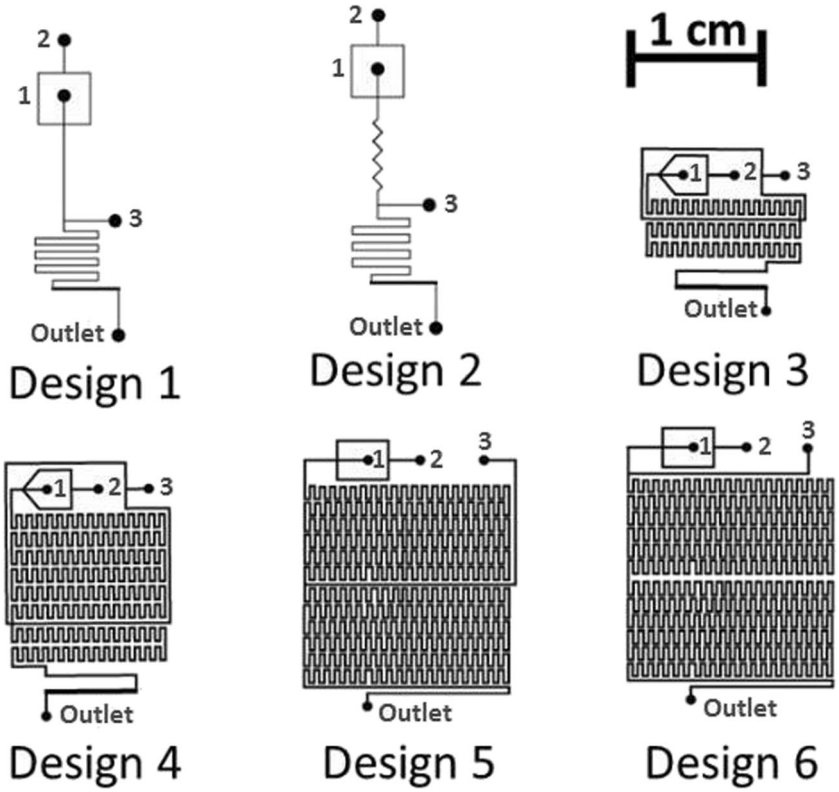

Fig. 1 Used manifolds to construct the microdevices which were evaluated to obtain the best extraction efficiency of polyphenols from extra virgin olive oil. Width and depth of the channels for designs 1 and 2 were 100 × 100 μm, respectively, and for designs 3 to 6 around 200 × 100 μm

microchannel picture in a microscope (Leica) with a 20× objective. First, the microchips were cut before being glued on glass, and transversal sections were observed measuring width, depth, and length of the microchannels with a total of 20 measurements per microchip using the ImageJ and PCC v2.7.756.2 software.

Microfluidic Liquid–Liquid Extraction Optimization

Preliminary Tests

The calibration of the syringe pumps was checked using distilled water and measuring in triplicate the water dispensed for 1 min at a flowrate of 40 μL/min.

The optimization study was carried out using different microchips (Fig. 1), according to previous designs developed in our working group (Sandoval-Ventura et al. 2017).

For the first experiments, the microchip design 1 in Fig. 1 was used to check if the extraction of polyphenols could be carried out. Extra virgin olive oil (2.0 g diluted at 5 mL with hexane), 0.5 mol/L NaOH, and 0.2 mol/L Folin–Ciocalteu reagent were used. All solutions were propelled into the microdevice via the peristaltic pump at 10 μL/min. A selection valve was also used for introducing different volumes of olive oil sample diluted in hexane (1.7, 3.4, and 5.0 μL). Two optical fibers were introduced to the output of the microchip in order to be able to measure the reaction product, but, due to that the separation of the mixture between the organic phase and the aqueous phase was not complete and an emulsion was formed, it was decided to do the extraction and the derivatization reaction inside the microchip and the spectrophotometric detection outside the microchip. In the outlet, a small piece of Teflon tubing connected the microchip with a 1-mL vial, where the reaction product (aqueous phase) and EVOO were collected. A dropper was used to separate the upper phase corresponding to olive oil, and, then, the spectrophotometric detection from the aqueous phase was performed outside the microchip using a Z-shaped flow cell with 400 μm optic fiber. The reaction product was introduced into the flow cell during 18 s employing a peristaltic pump (Ismatec) and Tygon tubing (Ismatec, internal diameter 0.76 mm) at a flowrate of 1 mL/min. Once filled, the flow was stopped, and the absorbance was measured at 730 nm for 4 min using distilled water as blank solution. Due to that the recorded signals were plateaus, an average value of the last 20 s of the absorbance values was used. All analyses were carried out in triplicate.

Complete Factorial Design

A 2^4 complete factorial design was constructed for beginning the optimization of the extraction process in the microchip. The parameters evaluated were flowrate, NaOH concentration, and Folin–Ciocalteu reagent concentration, and the prototypes were identified as design 1 and design 2, as shown in Fig. 1. A solution of extra virgin olive oil at 2% in hexane was used for the tests of the design of the experiments. Each evaluated factor was assigned a high (+) and a low (−) value (Table 1), and, with the help of the Statgraphics Centurion XVI.I software, the design matrix was built, making a total of 16 experiments. Folin–Ciocalteu reagent and NaOH solution were introduced into the microchips through inputs 3 and 1, respectively, and the sample through input 2.

The outlet of the microchip is plugged in with a Teflon tubbing to a vial to collect organic phase and aqueous phase.

Study of the pH Influence

The extraction of polyphenols from oils is principally carried out using methanol. In order to avoid this solvent, the extraction of polyphenols towards the aqueous phase must be in an alkaline medium so that the polyphenols are deprotonated and can be transferred from the organic phase to the aqueous phase; pH value in the aqueous phase, therefore, is one of the parameters most important for good extraction.

So, employing design 2 in Fig. 1 and a flowrate of 40 μL/min for the sample and reagents, three pH values (9.0, 10.6, and 13) and varying Folin–Ciocalteu reagent (Merck) concentrations (0.20, 0.04, and 0.02 mol/L) were evaluated. To fit the pH value, 0.1 mol/L carbonate buffer solution for pH 9.0, 0.1 mol/L carbonate buffer solution for pH 10.6, and sodium hydroxide for pH 13 were used. When the pH was fitted, different concentrations of carbonate buffer (0.10, 0.25, 0.50, and 0.75 mol/L) were also studied to evaluate how ionic strength influenced the extraction. For this study, 2% EVOO/hexane solution was continuously introduced through input 2. The reaction product was measured after 5 min of recollecting it into a 1 mL vial at the exit of the microchip.

Effect of Flowrate on the Extraction of Polyphenols

When the extraction of polyphenols is carried out in a microchip, flowrate is one of the most relevant hydrodynamic parameters in that process. It must be considered that in

Table 1 Values employed to build the 2^4 randomized factorial design for evaluating the principal factors for the extraction of polyphenols of extra virgin olive oil

Factor	Level	
	+	–
NaOH (mol/L)	0.5	0.05
F-C reagent (mol/L)	0.2	0.04
Flow (μL/min)	40	10
Microchip design	2	1

microfluidics the flowrates used in biochemical analysis are not very large (μL/h), but the microchip that is being evaluated will be applied for the extraction and quantification of polyphenols in food samples, and it is possible to use larger flowrates (μL/min) to increase the contact surface between oil and carbonate buffer. So, the microdevice marked as design 2 in Fig. 1 was used to evaluate the extraction flowrates, which values were both for reagents and samples—20, 30, and 40 μL/min with collection times at the vial exit of 10.0, 7.5, and 5 min, respectively. A solution at 8% of EVOO in hexane, a 0.75 mol/L pH 10.6 carbonate buffer solution, and a 0.02 mol/L Folin–Ciocalteu reagent solution were used for this study. The absorbances recorded for each flowrate were analyzed with the software Statgraphics, and, by means of an analysis of ANOVA, for comparison of multiple samples, it was determined if there were significant differences between the response variables.

Evaluation of the Chemical Kinetics of the Reaction Product

The derivatization reaction of the Folin–Ciocalteu reagent with polyphenols gives a complex blue color, whose intensity is proportional to the concentration of phenols and whose reaction kinetics, under batch conditions, is slow. Therefore, to ensure that the measurement of the reaction product outside the microchip was reproducible, the reaction mixture was collected in a vial for 5 min, the organic phase was removed, and a Z flow cell was filled the reaction product, which was in the aqueous phase, and its absorbance was measured for 90 min. For these experiments, 2% olive oil in hexane solution, 0.1 mol/L pH 10.6 carbonate buffer solution, and 0.02 mol/L Folin–Ciocalteu reagent solution and design 2 in Fig. 1 were used.

Study of the Length of the Microchannels of Extraction and Reaction

The liquid–liquid extraction efficiency is related to the contact time between the two immiscible liquids and solubility properties of the analyte. In this case, polyphenols in an alkaline medium are deprotonated and it is possible their extraction towards the aqueous phase. Also, in microfluidics, the contact surface is larger, helping the extraction besides if the time of contact between both phases increases the extraction too, and this could be achieved using larger coils. For this purpose, designs 2 to 6, shown in Fig. 1, which had different coil lengths, were evaluated. The designs had two parts; the first part corresponds to the extraction process where only the oil sample, diluted in hexane, and 0.75 mol/L pH 10.6 carbonate buffer solution are in contact; in the second part, the derivatization reaction between 0.02 mol/L Folin–Ciocalteu reagent solution and extracted phenols was carried out. In both parts, different lengths for the extraction coil and for the coil reactor were evaluated. Table 2 shows the physical characteristics for each design; width and depth of the channel, length of the extraction reactor (ECL), length of the reaction coil (RCL), and total volume into the coils (TV). The extracts and the oil samples were collected for 5 min, and, immediately thereafter, the aqueous phase was introduced into the flow cell measuring its absorbance for 1 h stopping the flow.

Evaluation of the Flow Behavior Within Microchannels

The extraction process was observed under the optical microscope (Leica) using the phantom camera (Ametek) at slow speed. The flow behavior between the two phases (aqueous and EVOO/n-hexane) was analyzed with principal interest in four zones of the microchannels: (1) mixing zone between 0.75 mol/L carbonate buffer solution at pH 10.6 and the EVOO/n-hexane solutions on inlet of the solutions, (2) extraction zone, (3) zone on inlet of the Folin–Ciocalteu reagent, and (4) reaction zone between Folin–Ciocalteu reagent and polyphenols.

Evaluation of the Microdevice Lifetime

Using design 4 in Fig. 1, which was selected to carry out the extraction of polyphenols from olive oil samples, the lifetime of the microchip was evaluated. The global processes of extraction and quantification are shown in Supplementary Material Fig. S1. The extraction of polyphenols was carried out in the first part of the microchip, where olive oil (input 2) and carbonate buffer solution (input 1) are introduced; then, Folin–Ciocalteu reagent was introduced through input 3; and, the reaction between polyphenols extracted and the reagent is carried out. Finally, the reaction product is collected in 1 ml vial and a flow cell was filled with the reaction product and measured at 730 nm.

Several extractions and measurements of polyphenols, from 8% EVOO solution in n-hexane were continuously carried out under optimal conditions for 8 h on a single day. At the end of the day, after several extractions, the microchannels were washed with distillated water–ethanol–distillated water for 15 min at 10 μL/min–10 μL/min–40 μL/min, respectively.

The analysis conditions for the extraction and reaction of polyphenols in olive oil were as follows: flowrate of 40 mL/min for reagents and sample, 0.75 mol/L carbonate buffer solution at pH 10.6, 0.02 mol/L Folin–Ciocalteu reagent, and collection time of 5 min into 1 mL vial. The aqueous phase was introduced into the flow cell measuring its absorbance in triplicate for 4 min, stopping the flow.

On the following day, using the same microchip, several extractions were carried out for 1 h. A comparison study was made between the signals obtained with the new microdevice and after being it used.

Validation of the Spectrophotometric Reaction for the Quantification of Polyphenols in Olive Oil

A daily calibration curve was prepared for seven days covering tannic acid concentrations in distillated water from 3.01 to 19.06 μg/mL (1.77 to 11.20 μmol/L). Under the reaction's optimal conditions using design 4, each standard was measured in triplicate. By input 2 were introduced the standards, by input 1 the pH 10.6 carbonate buffer solution, and by input 3 Folin–Ciocalteu reagent. The data were processed in the Statgraphics Centurion XVI.I software to construct a single calibration curve. The linear range as well as the limits of detection (LOD) and quantification (LOQ) were estimated according to the references from García et al.(2002) and Huber (2010).

Intralaboratory Repeatability and Reproducibility of Proposed Method

A 0.8% EVOO solution in hexane was prepared and measured, using a calibration curve daily constructed, in duplicate for seven days. Under the optimal conditions, triplicate extractions were performed for each solution, and, using the calibration curve, prepared daily, total polyphenols were quantified in the olive oil samples expressing the results in equivalent of tannic acid per kilogram of oil (mgETA/kg).

A one-way analysis of variance (ANOVA) was performed to determine the precision of the methodology (Miller and Miller 2004).

Batch Extractions

The results of the proposed microfluidic method were compared with those obtained using two batch extraction methods in accordance with Bendini et al. (2003) and the International Olive Council (IOC) (International Olive Council 2009). Two ratios of methanol/water, 60:40 by the Bendini method and 80:20 by IOC, were used as extractant solutions. A series of multi-extraction steps using a vortex mixer, ultrasonic bath, and centrifugation cycles were required. The extractions were done in triplicate for each sample, and, for the Bendini methodology, the quantification was done through the calibration curve built using the microdevice. For the other one, a batch calibration curve was constructed as follows: 1 mL of 0.75 mol/L carbonate buffer solution at pH 10.6 was added into a volumetric flask, followed by 1 mL of 0.02 mol/L Folin–Ciocalteu reagent, the necessary volume of tannic acid standards in methanol/water 80:20 *v*/*v* to cover a concentration range from 1.00 to 2.50 μg/mL, and 80/20 (*v*/*v*) methanol/water solution was employed to reach a total volume of 5 mL. The reaction could proceed for 40 min, and, thereafter, the absorbance was recorded at 730 nm in triplicate using 80:20 *v*/*v* methanol/water as blank.

Evaluation of Extraction Efficiency of the Evaluated Methods

Because the tannic acid is not soluble in hexane, a different phenol that was sufficiently soluble in hexane and that under the conditions of the microchip test could be deprotonated in alkaline medium and extracted into the aqueous phase was probed; thus, the extraction efficiency from the three extraction methodologies was evaluated using known concentrations of 2,4-dichlorophenol (Sigma-Aldrich) diluted in n-hexane, in accordance with the used extraction methodology, obtaining the following concentrations: 20.00 μg/mL for the microchip method (microchip 4 in Fig. 1), 232.65 μg/mL for the Bendini method, and 465.30 μg/mL for the IOC method. The quantification after each extraction process was referred to equivalent milligrams of tannic acid per liter of solution (mgETA/L). The percent of extraction efficiency was calculated by dividing the found concentration between the real concentration and multiplying the quotient by 100.

Batch extraction was also carried out in oil of the Pons Tradicional brand using a 0.75 mol/L pH 10.6 carbonate buffer solution. A total of 4 g of olive oil was weighed and diluted with 1 mL of hexane, and 2 mL of the carbonate buffer solution was added to stir for 2 min. Once finished, 6 drops of concentrated HCl were added to have an acidic pH (close to 2) in the extract and to prevent the polyphenols from being degraded. The mixture was centrifuged, and the aqueous phase was separated from the oil. Two more extractions were applied to the oil following the same procedure. All the aqueous phases were combined and filtered through an acrodisk. The resulting extract was transferred to a 10-mL volumetric flask and brought to the mark with water. The quantification of polyphenols was carried out with the same procedure as for the other two batch methods.

Another experiment that was carried out to evaluate efficiency was the extraction using the same design 4 of the microchip in Fig. 1 but with a mixture of 60:40 (*v*/*v*) methanol:water. In channel 2, the oil diluted in hexane (2.0 g/5 mL of hexane) was incorporated, and, in channel 1, the methanolic mixture entered. For this test, the Folin–Ciocalteu reagent mixture was not incorporated; so, channel 3 was closed. The mixture was collected in a vial, and the methanolic phase was separated to measure the concentration of polyphenols with the same procedure used as for the IOC quantification method. The same procedure was carried out to extract, in the microchip, polyphenols from olive oil using 0.75 M pH 10.6 carbonate buffer without adding the Folin–Ciocalteu reagent.

HPLC-DAD Study of the Extracts Obtained by the IOC Method and the Proposed One

For this study, the extraction was carried out in the microchip of an olive oil with 0.75 mol/L pH 10.6 carbonate buffer solution without introducing into the microchip the F–C reagent. At the

exit of the microchip, the extract was collected and separated from the oil and the pH of the aqueous solution was adjusted to 2 with concentrated HCl. On the other hand, a batch extraction was also carried out by the IOC method. Liquid chromatography was done under the following conditions: column Chromolith RP-18e 100–4.6 mm; sample injection volume 10 μL; temperature 25 °C; flowrate 1 mL/min; pressure 200 bar. Mobil phase: (A) water/acetic acid 99.8:0.2 *v*/*v* and (B) methanol. Concentration gradient A:B; 0 min 87/13; 7 min 87/13; 16 min 60/40; 22 min 60/40; 23 min 87/13.

Results and Discussion

Microchip Characterization

The characterization was carried out in the microdevices shown in Fig. 1. The microchips were made up of the following three main parts: (a) extraction zone, where the solution of EVOO in n-hexane and the carbonate buffer solution are introduced, ranging from its mixing to the incorporation of the reagent Folin–Ciocalteu; (b) reaction zone, where the Folin-Ciocalteu reagent is introduced into the microchip for the formation of the reaction product; and (c) outlet of the microdevice, for reaction product collection in a vial.

A comparative study between the acetate mask and the PDMS microchips showed that there was no any kind of imperfections or small stains that could be transferred from the mask to the final microchip. The microchannels were well-defined and without irregularities (see Supplementary Material Fig. S2). The results of the measurements of width, depth, length, and total volume of microchannels are summarized in Table 2 with their respective standard deviations. Cross-sectional view under the microscope can be seen in Supplementary Material Fig. S3.

Microfluidic Liquid–Liquid Extraction Optimization

Evaluation of Factorial Design

The extraction methodology of the phenolic fraction is based on polyphenol deprotonation in basic medium by exceeding their pKa value to form phenoxide ions, which, when presenting a charge, have a higher affinity towards the aqueous phase (pH 10.6 0.75 mol/L carbonate buffer solution). Based on this principle, the principal hydrodynamics and chemical parameters were evaluated using the factorial design described in Table 1. The results of the design were through ANOVA using the Statgraphics software aiming to maximize the analytical response (absorbance). A regression equation coded in terms of − 1 and + 1 was obtained with which the theoretical values of the experiments carried out were predicted. Subsequently, the residuals (difference between the predicted value and the observed value) were analyzed according to the number of experiments without observing any type of trend that would indicate systematic errors (Supplementary Material Fig. S4). When the standardized pareto diagram (Supplementary Material Fig. S5) was analyzed, it was determined with a level of significance of 95%, indicating the factors positively or negatively influence the extraction. All factors above the line perpendicular to the bars are statistically significant. In this case, the only significant factors were the concentrations of the Folin–Ciocalteu reagent and sodium hydroxide, reaching the maximum absorbance when the concentration of sodium hydroxide is the highest (+) (pH close at 13) and the Folin–Ciocalteu reagent is less concentrated (−). According to the design, the optimum conditions were as follows: 0.5 mol/L for NaOH and 0.04 mol/L for the Folin–Ciocalteu, reagent; however, turbidity was observed in the collected aqueous phase, which was more noticeable when the sodium hydroxide concentration increased, possibly either to the saponification process of the fatty acids in the olive oil in very alkaline medium or the stability of polyphenols in this medium. For the following experiments, the pH and the composition of the alkaline solution were modified, testing carbonate buffers, with the purpose of eliminating turbidity from the aqueous phase, since this is an interference causing overestimation of the absorbance values.

Influence of the Extraction pH

Studies have shown that extractant acidification with hydrochloric acid increases the amount of quantified phenolic compounds; however, when sodium hydroxide is used to alkalize

Table 2 Characterization of the coils from the six microdevices studied shown in Fig. 1

Design	Width (μm)	Depth (μm)	ECL (cm)	RCL (cm)	TV (μL)
1	95.54 ± 2.22	91.18 ± 6.29	0.75	2.60	0.46
2	96.78 ± 1.97	94.45 ± 7.24	0.97	2.60	0.50
3	211.23 ± 4.76	104.37 ± 6.18	4.63	10.74	4.53
4	219.42 ± 5.63	100.46 ± 7.63	26.36	10.75	9.66
5	196.743 ± 4.59	110.39 ± 6.19	34.22	36.19	16.27
6	201.48 ± 3.45	106.872 ± 8.29	70.57	70.57	15.85

ECL, extraction coil length; RCL, reaction coil length; TV, total volume

the extractant, the formation of precipitates is unavoidable due to the low stability of the polyphenols in strongly basic medium (Chethan and Malleshi 2007). The results of the 2^4 complete factorial experiment design showed that the pH and the composition of the alkaline solution could be caused by turbidity in the extracts. With the aim of eradicating it, a series of qualitative experiments was carried out. It is important to emphasize that the extraction was not carried out at acidic pH values because polyphenols are more soluble in aqueous medium when they are deprotonated and since the Folin–Ciocalteu assay requires an alkaline medium; in this way, it was possible to put together the extraction and the derivatization reaction in the same device.

When performing the extractions under the three different pH values (9.0, 10.6, and 13.0) and 0.20 N Folin–Ciocalteu reagent, there was no appreciable development of the reaction product from the extracted polyphenols, which does not imply that the phenolic fraction is not being extracted but rather that the Folin–Ciocalteu reagent is so concentrated that the yellow coloration predominates over the little product formed. When the concentration of the reagent is reduced to 0.04 N, improvement in the appearance of the blue color is noticeable, obtaining greater coloration at pH 10.6; however, the tonality of the extraction product is not completely blue (blue-green), which indicates that the concentration of the Folin–Ciocalteu reagent solution remains an interferer in the final coloration. At 0.02 N Folin–Ciocalteu reagent and using pH 10.6 carbonate buffer, it was possible to eliminate the yellow coloration of the Folin–Ciocalteu reagent on the reaction product, showing a completely blue shade. After determining that at pH 10.6 good results were obtained, the concentration of the buffer was evaluated. The increase in concentration and, therefore, in the ionic strength of the carbonate buffer solution affected positively the extraction of the phenolic fraction. The highest response was obtained at a concentration of 0.75 mol/L. Observing this behavior, attempts were made to prepare solutions at a higher concentration in order to improve the extraction; however, sodium carbonate reached its maximum solubility in water. Thus, the concentration 0.75 mol/L was selected as the optimum concentration. Therefore, the best results were obtained when the extraction in the microchip (design 4) was carried out using a buffer solution of 0.1 mol/L pH 10.6 carbonate and a 0.02 N Folin–Ciocalteu reagent solution, thus achieving to eliminate completely turbidity.

Study of the Length of the Extraction and Reaction Microchannels

By increasing the length of the extraction coil, the analytical signal increased; thus, the extraction is favored the longer contact time between the carbonate buffer solution and the olive oil solution. This behavior was noticed when the microdevices with designs 2 to 4 were evaluated. When analyzing the microchips from 4 to 6, it is noticed that, despite the difference in the length of the microchannels and although Folin–Ciocalteu reagent is introduced at the same time than the extractant reagent (design 6), there is no proportional increase in the response, suggesting the maximum extraction in the microchip has been reached from microchip 4, which was chosen to carry out the extraction and reaction of polyphenols from the extra virgin olive oil. In Supplementary Material Table S1, the width, depth, and length of the channels of the extraction and reaction parts are included. The extraction time of 3.86 s and the reaction time of 1.78 s obtained a global time of extraction and reaction of 5.64 s; so, although polyphenols are not stable in alkaline medium, the extraction time is so short that the decomposition of polyphenols is not possible, besides, immediately after the extraction, the derivatization reaction with the F–C reagent is carried out.

The kinetics of the reaction was also known by means of this study. Absorbance gradually increased 0.1 unit for 1 h of measurement; however, on the first 4 min, the signal remained stable, forming a small plateau. So, it was decided to introduce the reaction product into the flow cell immediately after completion of the collection time in the vial and measure the analytical signal for 4 min. Thus, it was possible to obtain reproducible analytical signals (see whole procedure in Supplementary Material Fig. S1).

Evaluation of the Microdevice Lifetime

The evaluation of the lifetime of the PDMS microdevice was indispensable because the material presents solubility and tends to swell in the presence of organic solvents, such as n-hexane. The foregoing involves a deformation of the microchannels or the presence of dissolved polymer oligomers that could directly affect the quantification (Lee et al. 2003).

According to the experimental results, it is concluded that a new microdevice can be used for at least eight continuous hours in a single day for different olive oil samples without presenting great variations in the recorded signals. However, after its use in one day, and although the microchannels had been washed, it was not possible to use it for further analysis, since the aqueous phase, where the reaction product was, had a slight turbidity altering the analytical response (Supplementary Material Fig. S6). Therefore, a new microchip was used every day.

Flow Behavior Within Microchannels

The microdevices have low Reynolds number values, which means that the flow is ordered (laminar flow) (Chakraborty 2010); however, Thorsen et al. demonstrated in 2001 that the interaction between two immiscible fluids leads to instability of the flow and, although the system allows low values of

Reynolds number, the flow is not laminar due to the competition between the surface tension and the shear force at the border of two non-static fluids, leading to the formation of droplets (Thorsen et al. 2001). The shape of the microchannels and the pressure with which the aqueous and oil phases are injected directly influence the size, distribution, and morphology of the drops, forming from monodisperse drops (pearl necklace) to emulsions (drops with zig-zag shape) (Squires 2005). This last behavior was observed along the microchannels of the microdevices studied, varying the length of the drops in the different sections of the microchannels. In Supplementary Material Fig. S7, the behavior of the flow in the microchannels in the different sections of the device is shown. In the intersection between the carbonate buffer solution and the olive oil sample in n-hexane, a clogging of the aqueous phase flow is observed by the oil solution, which causes an elongation in the flow, and, due to the high shear force located at the leading edge of the oil solution, the formation of microdrops is imminent (Thorsen et al. 2001), but the microdrops formed are not monodisperse, due mainly to the selected flowrate, since it has been observed that at flows of the order of μL/h a better control of its size is obtained (Squires 2005). The microdrops are clearly distinguishable and are maintained at the beginning of the extraction coil; however, the shape of the microchannels causes instability and competition between the shear force and the surface tension between the two immiscible phases leading to the formation of an emulsion (Thorsen et al. 2001). While both phases run through the microchannels, the emulsion is more evident. Folin–Ciocalteu reagent enters both ends of the central microchannel, and, due to the hydrophobic character of the PDMS, it is incorporated into the emulsion being maintained until the outlet of the microdevice.

Several experiments have shown that the microscale extraction and the formation of an emulsion improve the extraction due to the increase in surface/volume ratio, increasing mass transfer (Tsaoulidis and Angeli 2015; Woitalka et al. 2014).

Characteristic of the Quantification Method

The found linear range was from 1.18 to 11.79 μmol/L (2.00 to 20.00 μg/mL), and the equation was $A = 0.0155(\pm 4 \times 10^{-4})$ (tannic acid) $- 0.0142$ $(\pm 1 \times 10^{-3})$, $R = 0.9993$. The LOD was calculated considering a signal-to-noise ratio of the standard 3:1, while, for the LOQ, the ratio was 10:1, obtaining a concentration of 0.51 and 1.18 μmol/L, respectively. In Supplementary Material Fig. S8A, the calibration curve is shown, constructed with 95% confidence range, used for quantification of the extra virgin olive oil samples.

Intralaboratory Repeatability and Reproducibility

Precision is a measure of the similarity between the results obtained from the repeated application of the analytical method under the same conditions, and it can be determined in different ways. An intralaboratory repeatability (variation of the polyphenol concentration within the same day using the same sample, equipment, and analyst) and reproducibility study (variation of concentration between different days, using the same sample, equipment, and analyst) was carried out to determine the precision of the overall method (extraction and quantification).

A repeatability value of 1.45% and an intralaboratory reproducibility of 2.93% were obtained. Both percentages are less than 3.0% with very little variation and high precision.

Analysis of Extra Virgin Olive Oil Samples

Once it was demonstrated that the microdevice was suitable for the extraction of polyphenols from olive oil, as well as for them to react continuously with the Folin–Ciocalteu reagent for its quantification, different oil samples were analyzed, and the results were compared with two methods in a batch. The reaction products of the extracts, obtained by the methodology using the microchip and Bendini et al. (2003)), were quantified using the calibration curves of tannic acid in aqueous medium. Due to the statistically significant differences between the analytical signals of a tannic acid standard in aqueous medium and in methanol/water 80:20 *v*/*v*, the phenolic fraction extracted following the method described by the IOC was quantified with a tannic acid calibration curve in methanolic medium (Supplementary Material Fig. S8B).

The results of the quantification of total polyphenols of the six EVOO samples by the three methods are shown in Table 3. As can be seen, the concentration of phenolic compounds varies depending on the extraction method employed. The highest concentration was recorded when the microdevice was used, followed by the extraction suggested by the IOC, and the one recommended by Bendini et al.

If it is considered that the amount of extracted total polyphenols using the microdevice is 100%, then the percent extracted in the microchip was higher at 67 and 46%, on average, compared to Bendini et al. and IOC, respectively. This great difference in extraction can be attributed to several factors. First, we must consider the diversity of compounds constituent of the phenolic fraction in olive oil, e.g., phenolic acids, phenolic alcohols, flavonoids, lignans, and secoiridoides (the latter two being the most abundant compounds). Due to its chemical structure and independently of the methodology, the extraction will always be incomplete, leaving a certain amount of phenolic compounds retained in the organic phase. In this way, polyphenols can be subdivided into extractables and non-extractables. Derivatives of benzoic

Table 3 Total polyphenol concentration in extra virgin olive oils obtained from the proposed microdevice method and by two batch methods. From International Olive Council, IOC, (2009) and Bendini et al. (2003)

EVOO sample	Total polyphenols (mg of tannic acid/kg of oil) ($n = 3$)		
	Microdevice extraction carbonates	Bendini et al. extraction	IOC extraction
Sierra de Gata (Spain)	299.50 ± 3.05	102.65 ± 5.23	156.75 ± 5.98
Casas Hualdo, *Cornicabra* (Spain)	415.74 ± 6.39	138.19 ± 11.26	233.65 ± 7.97
Las doscientas Blend, *Arbequina/Picual* (Chile)	293.15 ± 7.35	101.69 ± 5.36	148.86 ± 8.11
Las doscientas, *Picual* (Chile)	357.67 ± 10.04	108.84 ± 3.72	163.91 ± 10.78
Pons Tradicional, *Frutado* (Spain)	245.69 ± 10.23	75.53 ± 3.12	130.27 ± 2.47
Pons Ecológico, *Natural* (Spain)	264.19 ± 11.49	85.91 ± 3.85	168.87 ± 15.63

acid and cinnamic acid can be an example of non-extractables, which form more complex structures by glycosidic bonds (hydrolyzable polyphenols). These compounds are retained in the extraction residue, and they are not quantified, producing an underestimation of the total polyphenol content (Godoy 2013).

Based on previous studies of recovery of non-extractable polyphenols in food matrices (Kim et al. 2006), the most common way of extracting them is through the use of either acidic or basic hydrolysis reaction to release most of the compounds associated with polysaccharides or linked together to form high molecular weight complexes. This demonstrates the importance of the nature of the extractant (methanol/water ratio and pH).

For batch extraction methods, the extractant consists of methanol/water mixture with an average pH value of 5.85 and 5.98 for the proportions 60:40 and 80:20 *v*/*v*, respectively. The pH for both extractant solutions is practically the same; so, the differences in the quantification can be attributed to the amount of methanol, favoring the extraction when methanol ratio is higher.

In the case of the microdevice extraction method, the extractant used was a 0.75 mol/L carbonate buffer solution at pH 10.6, achieving the extraction of the phenolic compounds mainly at the pH imposed. This criterion is extremely important, since when working with a mixture of solvents, such as methanol/water, the extraction pH maintains the polyphenols in their molecular form, whereas, when the pH is alkaline, the ionic form of phenolic compounds increases the affinity towards the aqueous phase, favoring its extraction.

Studies have shown that around 8 to 9% of extractable polyphenols (preferably free phenolic acids) are recovered from extraction with only methanol/water and more than 90% of hydrolyzable polyphenols linked to glucose are retained in the oil (Kim et al. 2006).

In this sense, the proposed methodology suggests that the use of a basic pH extractant solution can cause the hydrolysis of some compounds present in the oil, releasing the aglycone from oleuropein or ligustroside, greatly increasing the final quantification of total polyphenols. Likewise, the extraction could have been favored even more if the temperature factor had been considered during the experimentation, since when it is carried out under conditions of reflux, between 80 and 90 °C, the extraction is benefited (Chethan and Malleshi 2007).

Another aspect to consider is the sequence and number of steps to perform the extraction. For Bendini et al., the use of vortex mixer and phase separation were repeated in triplicate, which could cause imminent losses each time the extract was isolated. In the IOC methodology, the aqueous phase separation is carried out once; so, the loss of phenolic compounds is lower. However, it must be kept in mind that it was necessary to filter the extracts before being quantified; this extra step could directly decrease the amount of total polyphenols. The microdevice has the advantage of not being a multi-step extraction method, which implies lower analyte loss. In addition, working in a device with the diameter of channels in the order of microns to perform the extraction has the advantage of increasing the surface/volume ratio; thus, the area of contact between the EVOO sample in n-hexane and the carbonate buffer solution is amplified because of the formation of many microdroplets within the channels, increasing the transfer of mass.

In order to demonstrate that the physicochemical conditions of the microchip improve the extraction process of polyphenols from olive oil, a batch extraction using the same conditions as in the microchip was carried out.

The initial results showed that the extract obtained in batch at alkaline pH decreased the concentration as time passed; so, after 60 min, no signal was obtained. To prevent the polyphenols from degrading, pH was adjusted to 2 with concentrated HCl stabilizing them for more than 24 h. This was also demonstrated by HPLC obtaining a chromatogram without the signals corresponding to polyphenols in an alkaline extract after 2 h. However, in the extract adjusted to pH 2, the chromatogram had the presence of phenols and the chromatographic profile was very similar to that obtained in 80:20 methanol:water extracts (Fig. 2). These results show why the

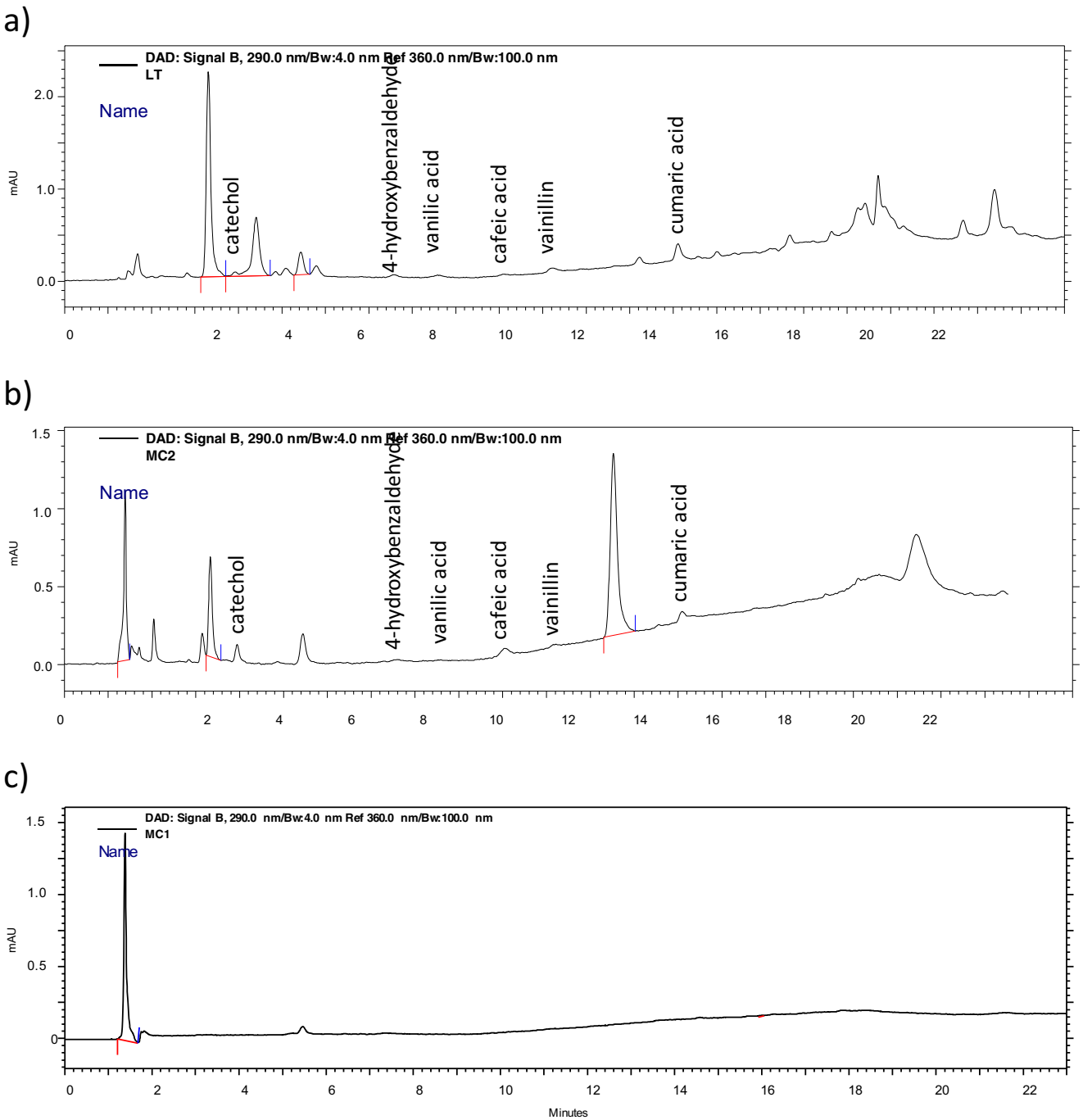

Fig. 2 Chromatograms for polyphenols obtained from (**a**) batch extraction with 80:20 methanol:water mixture; (**b**) microchip extraction with 0.75 M pH 10.6 carbonate buffer and later the extract adjusted at pH 2 before the injection; (**c**) microchip extraction with 0.75 M pH 10.6 carbonate buffer and later the extract injected in the chromatograph at pH 10.6

batch extraction is done in methanol, since polyphenols are not stable in an alkaline medium. However, in the microchip, the polyphenol analysis time is very short, in the order of seconds; so, polyphenols do not reach to degrade. This is in addition to favoring the equilibrium towards the aqueous phase because at the same time that the extraction is carried out the polyphenol oxidation reaction is taking place; so, due to the law of mass action, the equilibrium moves towards the aqueous phase, improving the extraction of polyphenols.

Extraction Efficiency

The tannic acid standard did not show solubility in n-hexane or olive oil sample, and it was not suitable for estimating with

Table 4 Results of the extraction efficiency for the three extraction methods evaluated using a 2,4-dichlorophenol (2,4-DCF) standard solution

Extraction method	Extraction number	2,4-DCF (mgETA/L)		% Extraction efficiency	% Extraction efficiency ± S (n = 3)
		Added	Found		
Microdevice	1	20.01	8.06	40.27	39.90 ± 0.68
	2		7.83	39.11	
	3		8.07	40.31	
Bendeni et al.	1	235.35	18.59	7.89	8.28 ± 0.46
	2		19.24	8.17	
	3		20.69	8.79	
IOC	1	465.3	78.33	16.83	15.40 ± 1.50
	2		64.33	13.83	
	3		71.66	15.40	

S, standard deviation; IOC, International Olive Council

it the efficiency extraction of the methodologies. In this case, it was decided to employ a standard of phenolic nature with solubility in n-hexane, selecting a 2,4-dichlorophenol (2,4-DCF) standard.

This study was carried out for the following two purposes: (1) comparing between extraction methodologies and (2) obtaining numerical values using a standard that supports the quantification results of total polyphenols from the olive oil samples.

The results obtained by the three methods are presented in Table 4. The extractions with the standard 2,4-DCP show the same behavior as the EVOO samples achieving the greater extraction with the use of the microdevice, followed by the extraction proposed by the IOC, and that of Bendini. However, this does not imply that only about 40% of the phenolic compounds are extracted with the microdevice, since the calculated percentages cannot be directly related to the extraction of the phenolic fraction, because the 2,4-DCP standard had more affinity towards the organic phase (n-hexane), implying that its extraction in the carbonate buffer solution will be much lower than that of the polyphenols contained in olive oil.

Also, in order to demonstrate that the extraction efficiency in the microchip is higher than that by the batch methods, the extraction of polyphenols was carried out by a batch method using carbonate buffer (the "Batch Extractions" section) and carrying out the extraction in the microchip using as extractant a methanol:water mixture (60/40) in accordance with the Bendini method (the "Evaluation of Extraction Efficiency of the Evaluated Methods" section). The test was carried out on the ecologic oil showing similar results; using the microchip, the extraction with methanol:water (60:40) was 217.81 ± 9.65 mg tannic ac./kg oil and with carbonates 264.19 ± 11.49 mg tannic ac./kg oil; for batch extractions in methanol:water (60:40) was 75.53 ± 3.12 mg tannic ac./kg oil and with carbonates was 68.81 ± 1.79 mg tannic ac./kg oil. These results show that the values using the Bendini method and the batch extraction with carbonate buffer are similar and that the concentration of polyphenols extracted depends on the extraction procedure, demonstrating that the extraction with the microchip is approximately between 50 and 60% more efficient.

Conclusions

Despite the incompatibility between the PDMS and the solvent n-hexane/olive oil, photolithography and soft lithography techniques proved to be useful for the rapid and mass construction of microdevices with duration of at least 8 h of use during a single day, which involves the accomplishment of about 80 extractions, obtaining repeatable and reproducible analytical signals.

It was possible to develop and optimize the liquid–liquid extraction of the phenolic compounds present in the EVOO samples, finding that the statistically significant factors in the extraction are pH and concentrations of the carbonate buffer solution and Folin–Ciocalteu reagent; in addition, the increase in the extraction coil length influences positively.

The quantification as total polyphenol content in extra virgin olive oil is expressed in mgETA/kg showed that the use of the microdevice is more efficient, extracting from 46 to 67% more polyphenols. This trend was corroborated by the extraction efficiency of the 2,4-dichlorophenol standard in n-hexane, which, although they cannot be directly related to the extraction of polyphenols from the olive oil sample, due to their nature and lower solubility in aqueous medium, serves to obtain a numerical value that supports the observed results. Also, the results of the batch and microchip extractions using as extractants carbonates or the methanol:water mixture showed that the extraction in the microchip is more efficient than in the batch independent of the extractant that is used.

HPLC assays showed that polyphenols extracted using carbonates as extractant are similar in composition than when a methanol:water mixture is used.

The microdevice has the advantages of requiring less sample and reagents, which means less waste generation. Likewise, there is minimal manipulation by the user and a short analysis time, with up to 10 extractions with quantification per hour (approximately three different samples of olive oil in triplicate in 1 h).

Acknowledgements The authors are truly grateful to Dirección General de Asuntos del Personal Académico (DGAPA) (Project Number IN218415) and to Facultad de Química of Universidad Nacional Autónoma de México (Programa de Apoyo a la Investigación y el Posgrado) (PAIP 5000-9029 and 5000-9023), for their contribution and financial support.

The authors are also grateful to Minerva Monrroy Barreto, PhD, from Unidad de Apoyo a la Investigación y a la Industria (USAII), Facultad de Química, for carrying out the HPLC analyses.

Compliance with Ethical Standards

Conflict of Interest Kenia Chávez-Ramos declares that she has no conflict of interest. Luis Fernando Olguín-Contreras declares that he has no conflict of interest. María del Pilar Cañizares-Macías declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

References

Bendini A, Bonoli M, Cerretani L, Biguzzi B, Lercker G, Gallina Toschi T (2003) Liquid-liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. J Chromatogr A 985(1–2):425–433. https://doi.org/10.1016/S0021-9673(02)01460-7

Bonoli M, Montanucci M, Toschi TG, Lercker G (2003) Fast separation and determination of tyrosol, hydroxytyrosol and other phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis with ultraviolet-diode array detection. J Chromatogr A 1011(1–2):163–172. https://doi.org/10.1016/S0021-9673(03)01100-2

Chakraborty S (2010) Microfluidics and microfabrication. Microfluidics and Microfabrication https://doi.org/10.1007/978-1-4419-1543-6

Chethan S, Malleshi NG (2007) Finger millet polyphenols: optimization of extraction and the effect of pH on their stability. Food Chem 105(2):862–870. https://doi.org/10.1016/j.foodchem.2007.02.0

García MA, Soberón E, Cortés M, Rodríguez R, Herrera JL, Alcántara A (2002) Guía de validación de métodos analíticos, 1st edn. A. C. Colegio Nacional de Químicos Farmacéuticos Biólogos México, Ed., Mexico

García-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A (2010) Phenolic-compound-extraction systems for fruit and vegetable samples. Molecules 15(12):8813–8826. https://doi.org/10.3390/molecules15128813

Godoy M d P (2013) Nuevas propuestas de procedimientos analíticos sencillos para la cuantificación de compuestos fenólicos presentes en la fracción minoritaria de aceite de oliva. Universidad de Extremadura

Huber L (2010) Validation of analytical methods, vol 2. Agilent Technologies

International Olive Council. (2009). Determinación de los biofenoles de los aceites de oliva mediante hplc

Kim KH, Tsao R, Yang R, Cui SW (2006) Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem 95(3):466–473. https://doi.org/10.1016/j.foodchem.2005.01.032

Kralj JG, Sahoo HR, Jensen KF (2007) Integrated continuous microfluidic liquid–liquid extraction. Lab on a Chip 7(2):256–263. https://doi.org/10.1039/B610888A

Lee JN, Park C, Whitesides GM (2003) Solvent compatibility of poly(-dimethylsiloxane)-based microfluidic devices. Anal Chem 75(23):6544–6554. https://doi.org/10.1021/ac0346712

Lim YC, Kouzani AZ, Duan W (2010) Lab-on-a-chip: a component view. Microsyst Technol 16(12):1995–2015. https://doi.org/10.1007/s00542-010-1141-6

Lozano J, Fernández-Gutiérrez A, Segura-Carretero A (2009) Composición del aceite de oliva. Aceite de Oliva Virgen: Tesoro de Andalucía 48:197–224

Maruyama T, Matsushita H, Uchida J, Kubota F, Kamiya N, Goto M (2004) Liquid membrane operations in a microfluidic device for selective separation of metal ions. Anal Chem 76(15):4495–4500. https://doi.org/10.1021/ac049844h

Mary P, Studer V, Tabeling P (2008) Microfluidic droplet-based liquid–liquid extraction. Anal Chem 80(8):2680–2687. https://doi.org/10.1021/ac800088s

MicroChem (n.d.) SU-8 3000 permanent epoxy. https://doi.org/10.1146/annurev.matsci.28.1.153

Miller JN, Miller JC (2004) Statistics and chemometrics for analytical chemistry. Technometrics 46:498–499. https://doi.org/10.1198/tech.2004.s248

Monasterio RP, De Los Angeles Fernández M, Silva MF (2013) High-throughput determination of phenolic compounds in virgin olive oil using dispersive liquid-liquid microextraction- capillary zone electrophoresis. Electrophoresis 34(12):1836–1843. https://doi.org/10.1002/elps.201300117

Pirisi FM, Cabras P, Cao CF, Migliorini M, Muggelli M (2000) Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. J Agric Food Chem 48PDF:1191–1196

Ríos Á, Zougagh M, Avila M (2012) Miniaturization through lab-on-a-chip: utopia or reality for routine laboratories? A review. Anal Chim Acta 740:1–11. https://doi.org/10.1016/j.aca.2012.06.024

Salik A, Fabek D, Rukavina I, Zelic B (2011) Aqueous two-phase extraction of polyphenols using a microchannel system—process optimization and intensification. Food Technol Biotechnol 49(4):495–501

Sánchez-Rangel JC, Benavides J, Heredia JB, Cisneros-Zevallos L, Jacobo-Velázquez DA (2013) The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. Anal Methods 5:5990. https://doi.org/10.1039/c3ay41125g

Sandoval-Ventura O, Olguín-Contreras LF, Cañizares-Macías M d P (2017) Total polyphenols content in white wines on a microfluidic flow injection analyzer with embedded optical fibers. Food Chem 221:1062–1068. https://doi.org/10.1016/j.foodchem.2016.11.055

Spietelun A, Marcinkowski Ł, De La Guardia M, Namieśnik J (2014) Green aspects, developments and perspectives of liquid phase microextraction techniques. Talanta 119:34–45. https://doi.org/10.1016/j.talanta.2013.10.050

Squires TM (2005) Microfluidics: fluid physics at the nanoliter scale. Rev Mod Phys 77(July):978–1016

Temiz Y, Lovchik RD, Kaigala GV, Delamarche E (2015) Lab-on-a-chip devices: how to close and plug the lab? Microelectron Eng 132:156–175. https://doi.org/10.1016/j.mee.2014.10.013

Thorsen T, Roberts RW, Arnold FH, Quake SR (2001) Dynamic pattern formation in a vesicle-generating microfluidic device. Phys Rev Lett 86(18):4163–4166. https://doi.org/10.1103/PhysRevLett.86.4163

Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S, La Guardia M, La Guardia M (2005) The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr Res Rev 18(1):98–112. https://doi.org/10.1079/NRR200495

Tsaoulidis D, Angeli P (2015) Effect of channel size on mass transfer during liquid-liquid plug flow in small scale extractors. Chem Eng J 262:785–793. https://doi.org/10.1016/j.cej.2014.10.012

Woitalka A, Kuhn S, Jensen KF (2014) Scalability of mass transfer in liquid-liquid flow. Chem Eng Sci 116:1–8. https://doi.org/10.1016/j.ces.2014.04.036

Zaouk R, Park BY, Madou MJ (2006a) Fabrication of polydimethylsiloxane microfluidics using SU-8 molds. Methods Mol Biol 321:17–21. https://doi.org/10.1385/1-59259-997-4:17

Zaouk R, Zaouk R, Park BY, Park BY, Madou MJ, Madou MJ (2006b) Introduction to microfabrication techniques. Methods Mol Biol 321:5–15. https://doi.org/10.1385/1-59259-997-4:3