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On line coupling of a liquid–liquid extraction flow-reversal system to a spectrophotometric flow-through sensor for the determination of polyphenols in olive oil

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Abstract

The coupling of a liquid–liquid extraction approach based on flow-reversal to a flow-through sensor is proposed. The basis of the sensor is the use of Folin–Ciocalteau reagent immobilized on an anionic exchange resin packed in a flow cell located in a spectrophotometer, where the reaction product (molybdenum blue) is formed, retained and detected on the resin surface, thus integrating the reaction, retention and detection. The regeneration of the sensor is accomplished by passing an acidic Ce(IV) solution through it. This coupled system allows the extracted analytes (polyphenols) to be monitored continuously, and it was used to develop a method for the determination of polyphenols in oil. The proposed method features two linear response ranges, from 15 to 30 and from 30 to 90 μg ml^{-1}, expressed as caffeic acid, with a relative standard deviation $< 3.1\%$. The 3σ detection limit was 10 μg ml^{-1} caffeic acid. It was applied to the determination of polyphenols in olive oil samples with results in excellent agreement with those provided by a conventional method.

Keywords: Flow system; Extraction; Spectrophotometry; Sensors; Polyphenols; Olive oil

1. Introduction

The automation of the analytical process promoted by the growing demand for rapid and safe analysis in the environmental, food, clinical and industrial areas has had a varied development. Preliminary operations [1] are claimed to be variable, complex, slow, prone to errors and difficult to calibrate. These features are the reasons why their automation has not been as spectacular as that undergone by the other steps of the analytical process, although there is no doubt about the importance of the preliminary operations in the quality of the analytical results, their intensive human participation and their potential hazards to both laboratory personnel and the environment.

Automated continuous-flow systems [2,3] have contributed largely to the development of preliminary operations, as the versatility of these systems makes it possible to automate non-chromatographic separation techniques such as precipitation, liquid–liquid extraction, gas-diffusion and dialysis, frequently involved in the first steps of the analytical process. Liquid–liquid extraction has been carried

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out in continuous-flow systems both with and without phase separation. The latter has two main advantages over the conventional dynamic method: the absence of a phase separator, a source of shortcomings of the separation procedure, and a substantial simplification of the continuous manifold. It can be implemented without passage of the interphase through the detector (by iterative inversion of the flow [4,5]) as well as with passage of the aqueous–organic interphase, a parasitic-signal elimination being required in this case [6,7]. The approach based on flow reversal permits both gradual enrichment of the interphase in the analyte and monitoring of the kinetics of the extraction process.

The development of physical and (bio)chemical sensors [8] also seems to be a promising approach to the automation of preliminary operations. Flow-through sensors integrating reaction and/or retention with detection via a suitable support packed in the flow cell of a conventional, non-destructive optical detector [9,10] have proved to be powerful tools for enhancing such basic analytical features as sensitivity and selectivity. Moreover, these kinds of sensors surpass the probe type used in batch measurements in several aspects, including its easier regeneration and calibration. The in situ concentration of the monitored product in a small area provided by these systems that integrate reaction, retention and detection, and dramatically lower the detection limits [11–13]. In addition, the immobilization of the reagent reduces its consumption, avoids the use of complex manifolds, and decreases analyte dilution.

In this paper, a sensor for the determination of polyphenols in olive oil based on the immobilization of the Folin–Ciocalteau reagent in an appropriate support packed in a flow cell is reported. The flow-through sensor is implemented in conjunction with a flow reversal approach that carried out the gradual on-line liquid–liquid extraction of the polyphenols simultaneously with their reaction, retention of the reaction product and detection in the sensor. Furthermore, the on-line coupling of the iterative flow-reversal system to the sensor enables the integration of preliminary operations, such as dilution, measurement of the sample volume and liquid–liquid extraction, and then the monitoring of the analytical signal, and so there is a direct connection between the raw sample and the analytical results.

2. Experimental

2.1. Instruments and apparatus

A Unicam 8625 UV-visible spectrophotometer equipped with a Hellma 178.12QS flow cell (18 μl inner volume, 1.5 mm optical path) connected to a Kipp chart recorder was used. A Gilson Minipuls-3 peristaltic pump controlled by a Commodore 64 computer allowed the start of the reversal cycles, the number of cycles (n) and the cycle duration (Δt) to be programmed. An Ismatec S-840 peristaltic pump and three Rheodyne 5041 low-pressure injection valves (two of them acting as switching valves) were also used. All PTFE tubing of the flow system was 0.7 mm inner diameter. The pump tubing used to pump organic solvent was Solvaflex (Technicon).

2.2. Reagents

All reagents were of analytical reagent grade. An aqueous solution of 1% (v/v) Folin–Ciocalteau reagent (Merck) was used. The reagent was immobilized on diethylaminoethyl–Sephadex anion exchanger (DEAE–Sephadex, bead size 40–120 μm; Sigma) packed in the flow-cell. An 0.1 M aqueous solution of $NaHCO_3$ (Merck) adjusted to pH 8.2 was used as carrier/extractant. An aqueous solution containing 500 μg ml^{-1} $(NH_4)_2Ce(NO_3)_6$ (Merck) and 0.5 M H_2SO_4 was also used. Calibration solutions of caffeic acid (Sigma) were prepared from a 1 g l^{-1} stock solution in ethanol by appropriate dilutions in n-hexane (Panreac). The olive oil samples were provided by the Olive growing Station of Mengíbar, Jaé (Spain) and they were diluted with n-hexane before analysis. A 1:1 (v/v) ethanol–diethylether solution was used to clean the system after each determination.

2.3. Immobilization and flow-cell packing

The DEAE–Sephadex anion exchanger was conditioned by treatment with distilled water before placing it in the flow cell, which was packed to such a level that the light beam would cross the upper part of the solid material when the flow cell was in the spectrophotometer (Fig. 1). Two glass-wool plugs were used in both ends to preserve the position of

the resin in the flow-cell, when the flow was iteratively reversed. The 1% Folin–Ciocalteau solution was passed through the packed resin at a flow-rate of 1.0 ml min^{-1} for 2 min, which was sufficient to saturate the resin. The sensor prepared under these conditions was used throughout this work (ca. 2 months) without any alteration in its performance. The reagent was permanently immobilized in the anion exchanger and no bleeding was observed when carrier was passed through the packed flow cell. The reaction product (molybdenum blue) also remained on the solid support even when several eluents were used in an attempt to elute it from the support. The strong retention of both the reagent and reaction product was not a shortcoming as it was possible to convert the reaction product into the reagent again by using an oxidant such as Ce(IV). After this easy regeneration the sensor is ready again for a next injection.

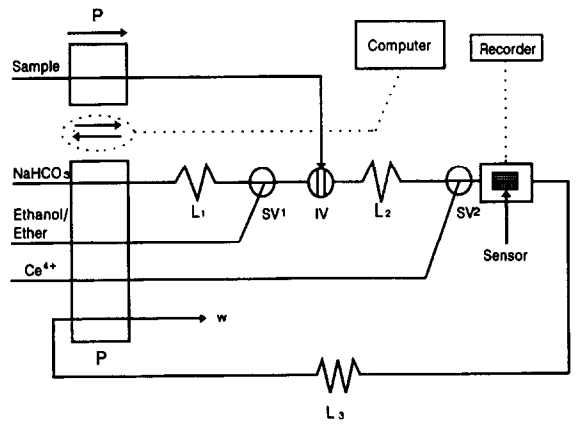

Fig. 2. Flow reversal/liquid–liquid extraction manifold-sensor arrangement for the determination of polyphenols. P, peristaltic pump; SV, switching valve; IV, injection valve; L, coil; w, waste.

2.4. Manifold and procedure

Fig. 2 illustrates the flow-injection manifold used. 500 μl of oil, previously diluted (1:5 sample/n-hexane volume ratio) is injected into the aqueous extractant acting as carrier. The iterative change of the flow direction is synchronized with the injection in such a way that the sample injected zone (organic phase) never reaches the sensor (thus avoiding parasitic signals) but arrives as close to it as possible, thus allowing the most analyte-enriched zone of the extractant phase and the sensor to come into contact.

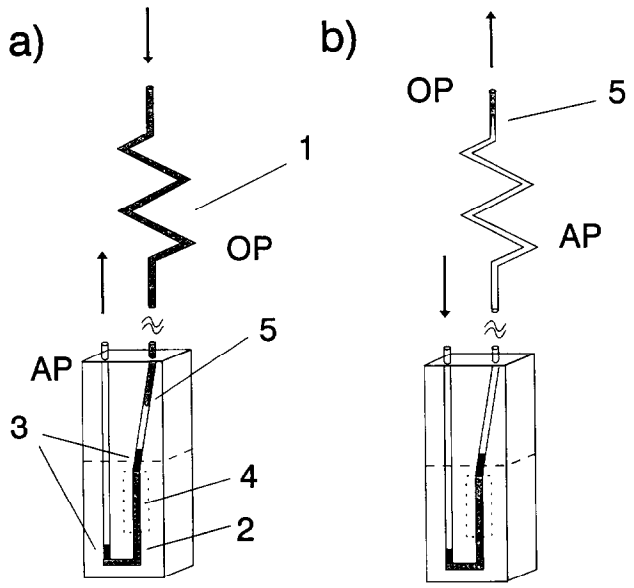

Fig. 1. Diagram of the packed flow cell arranged as the proposed sensor and the extraction coil. Extraction coil (1), packed DEAE-Sephadex anion exchanger with immobilized reagent (2), glass-wool plug (3), optical window (4), aqueous–organic interphase (5). OP and AP denote organic and aqueous phases, respectively.

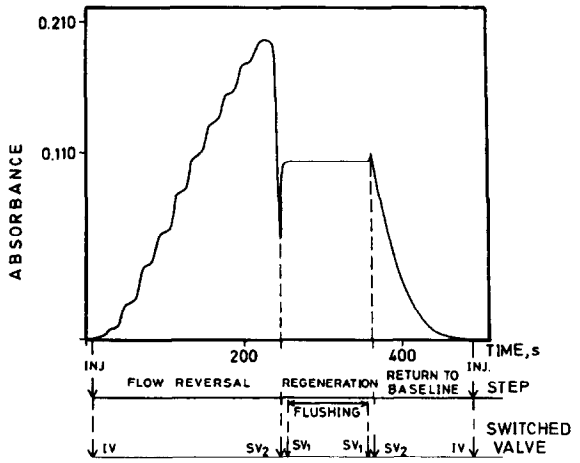

Fig. 3. Type of recording obtained with the proposed reversal-flow liquid–liquid-extraction system coupled to a sensor, and timetable of analysis steps.

The stream that leaves the sensor is aspirated to waste by the same computer-operated peristaltic pump in order to avoid differences in the flow-rate in the two directions. The coils L_1, L_2 and L_3 were used to prevent the solutions from returning to the reservoirs when the flow direction is reversed. The extraction of polyphenols from oil takes place along the continuous system by iterative changes of the flow direction. The aqueous phase continues growing richer in polyphenols in each cycle, which are then removed from that phase each time that it comes into contact with the resin packed in the flow cell. When the enriched extractant reaches the material packed in the flow cell in which the Folin–Ciocalteau is immobilized, the polyphenols react with it, the reaction product (molybdenum blue) remains on the resin and is monitored at 750 nm. The recording obtained (Fig. 3) does not exhibit the typical maxima and minima of conventional multipeak recording of a flow-injection system with iterative reversal of the flow direction. In the proposed system, the recording lacks minima because the monitored product is retained at the detection point (integration of reaction/retention/detection) and so the analytical signal does not decrease when the richer portion of the extractant (closer to the interphase) leaves the sensor.

When the preselected cycles are finished, valve SV_2 is switched thus allowing the passage of the Ce(IV) solution through the sensor for 2 min; 5 s later SV_2 is activated and SV_1 is switched to clean the system (from SV_1 to SV_2) with an ethanol–ether mixture. Once the system has been cleaned, SV_1 is switched 30 s earlier than SV_2 to avoid the ethanol–ether mixture reaching the sensor. SV_2 is placed as close to the sensor as possible in order to clean the largest portion of the flow system in contact with the organic phase.

3. Results and discussion

3.1. Type of support

Several types of support were investigated to immobilize the Folin–Ciocalteau reagent. As expected, the reagent was not remained on supports such as C_{18} bonded silica (Waters, Sep-pak, bed size 100 μm) or XAD (type 4 and 7, from Serva) due to its anionic nature. Anion exchangers showed very different behaviour; Dowex 2 did not retain the reagent while the retention on QAE–Sephadex was weak and the reagent was eluted slowly from the support when carbonate buffer solution was passed through. The reagent was permanently immobilized on DEAE–Sephadex and no bleeding caused by the carrier solution was observed.

3.2. Optimization of variables

The univariate method was selected for optimization purposes. The aim of the optimization was threefold to obtain an increased signal, to avoid the formation of an emulsion inside the flow system and to set up the best working conditions for the maintenance of the sensor.

3.2.1. Chemical variables

Sodium hydroxide and carbonate solutions were used as carriers in order to provide the alkaline medium required to favour the extraction of polyphenols and to accomplish the reduction of the Folin–Ciocalteau reagent. 0.1 and 0.01 M sodium hydroxide were rejected as carriers because they affected the sensor stability. This shortcoming was overcome by using 0.1 M sodium carbonate as carrier. The pH of the carbonate solution was studied in the range

7–10. An increased pH resulted in improved extraction but above 8.2 the baseline was higher after each regeneration step, so this value was chosen as optimum for further experiments. In this way, the formation of microemulsions, a source of irreproducibility with more alkaline media [14,15] was avoided.

Although the use of 60% methanol in the aqueous phase was reported in the conventional method to give better extraction of the analytes [14], and Mesa et al. [15] decreased this by up to 5% to avoid emulsion formation, in the flow-reversal system this organic solvent was omitted from the aqueous phase as it caused anomalous packing of the resin and irreproducibility of the signals provided by the approach reported here.

The dilution of olive oil with n-hexane decreased and homogeneized such an important feature of this system as sample viscosity, thus improving its hydrodynamic behaviour. In fact, the interval between cycles required by the interphase to reach the sensor proximity varied from 15 to 17 s when n-hexane or diluted olive oil, respectively, were used.

An 1:1 ethanol–diethylether solution has previously been reported as being useful for flushing the remaining oil or organic solvent from the system, once an analysis is finished. This mixture cleaned the system between SV_1 and SV_2 without passing through the flow-cell.

Since the retention of both the reagent and the reaction product on the anion exchanger was so strong that none of the potential eluents tested (NaCl, HCl, HNO_3, citric acid, NH_4Cl and NaOH) was effective, the regeneration of the Folin–Ciocalteau reagent by oxidation of the reaction product was studied. A strong oxidant such as Ce(IV) was required to restore the baseline in as short a time as possible after data collection. Anionic oxidants, which could progressively bind to the resin, partially displacing the reagent from it, were rejected. The effect of the concentration of $(NH_4)_2Ce(NO_3)_6$ was studied between 50 and 700 $\mu g\ ml^{-1}$; 500 $\mu g\ ml^{-1}$ was selected as lower concentrations needed more time to regenerate the sensor and higher ones gave rise to non-reproducible results. Under these working conditions, regeneration was achieved in 2 min. When the regeneration of the sensor was complete and the flow system was cleaned and filled with carrier/extractant solution, a flow-reversal step consisting of six short cycles (10 s each) was necessary in order to recover the original compactness and position of the resin packed in the flow-cell.

3.2.2. *Flow-injection variables*

The length of the extraction coil, L_2, was 100 cm. Other coils, L_1 and L_3, were large enough (150 cm) to avoid contamination of the carrier reservoir and the flow-cell, respectively, when the flow direction was reversed.

The flow-rate, number of cycles (n) and the duration of each cycle (Δt) were simultaneously studied because of their interrelation. This study was aimed at obtaining the maximal signal in the shortest time possible. The highest signal was obtained when maximal approach of the interphase to the sensor occurred. Contact of the interphase with the resin was always avoided. As the distance (tubing length) between the injection valve and the sensor was fixed, the flow-rate and the cycle duration could not be changed independently. The flow-rate was studied between 1.3 and 2.5 ml min^{-1} and the duration of the cycle ranged from 30 to 10 s. The influence of the flow-rate on the analytical signal for three different numbers of cycles is shown in Fig. 4, where the time required to obtain them is also indicated. The analytical signal increased with decreasing flow-rate so the lowest flow-rate (1.3 ml min^{-1}) was recommended. In addition, back pressure drawbacks were observed for flow-rates > 2 ml min^{-1}.

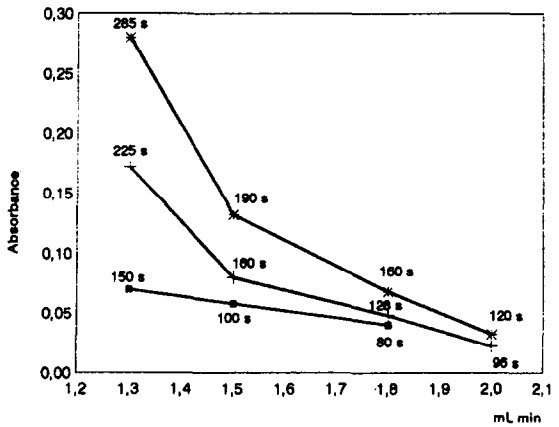

Fig. 4. Influence of flow-rate on analytical signal for 10 (■), 16 (+) and 20 (*) cycles. The analysis time for each case is also shown. Each cycle takes 15 s.

The determination time depends on the number of cycles and the time between them, which should be as short as possible. Increasing the number of cycles up to 20 resulted in increased sensitivity, but above this value the effect almost disappeared and the determination time became too long: thus a 20-cycle period was selected.

The influence of the injected volume on the analytical signal was studied in the range 200–700 μl. Increasing injection volumes up to 500 μl resulted in increased analytical signals but the effect was slight (< 5%) above this value, so a volume of 500 μl was chosen as optimum.

3.3. Kinetics and efficiency of the liquid–liquid extraction

The concentration of the reaction product at the detection point allowed the monitoring of the liquid–liquid extraction process. The kinetic curves of the liquid–liquid extraction for two concentration levels in the organic phase are shown in Fig. 5. They were obtained by summing the maximum signals of 30 successive cycles. As can be seen, these curves exhibit a short induction period for which the slope is smaller than those for longer times. Even at the beginning of the extraction the concentration of polyphenols in the organic phase is maximal and so the extraction should be faster. This behaviour could be due to the existence of a segment of the aqueous phase between the interphase and the sensor that never reaches the sensor and along which a gradient of polyphenols concentration must be established until the extracted polyphenols reach the sensor. After this period, from ca. 6 or 8 to 20 cycles, the rate of extraction (slope of the curve) decreased with time as a consequence of the decreasing analyte concentration in the organic phase along the extraction process. Finally, the extraction became very slow from 20 or 30 cycles, depending on the initial concentration of analytes in the oil. Ca. 50% of the analyte in the organic phase reached the sensor after 30 cycles. This recovery was calculated for a 20-μg ml^{-1} caffeic acid solution in n-hexane by comparing the signal with that obtained for the same concentration of analyte in sodium carbonate solution that was injected and led straight to the sensor without any reversal of the flow direction. Increasing the number of cycles above 30 did not result in increased recoveries. There seems to be a limit of 50% recovery

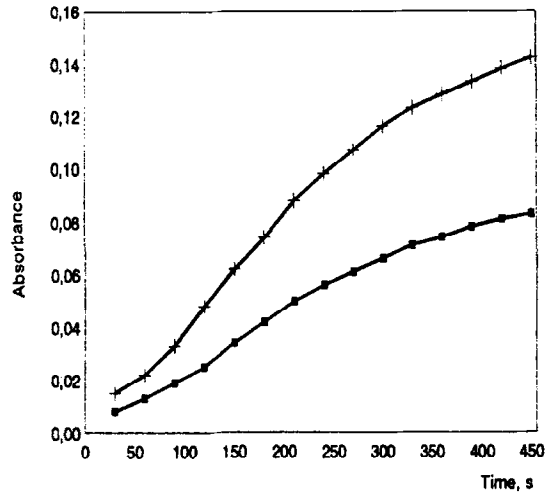

Fig. 5. Influence of the number of cycles on the analytical signal for 20 (■) and 50 (+) μg ml^{-1} caffeic act. Each cycle takes 15 s.

Table 1
Features of the proposed method

Number of cycles (n)	15–30 μg ml^{-1} [a]			35–90 μg ml^{-1} [a]		
	Calibration slope (ml μg^{-1})	r (n = 6)	r.s.d. (%) [b]	Calibration slope (ml μg^{-1})	r (n = 6)	r.s.d. (%) [c]
10	0.005	0.9969	5.1	0.0020	0.9990	5.2
12	0.006	0.9990	4.6	0.0023	0.9985	5.3
14	0.008	0.9890	4.0	0.0025	0.9908	4.2
16	0.010	0.9900	2.8	0.0025	0.9980	4.0
18	0.011	0.9930	2.8	0.0026	0.9920	3.2
20	0.013	0.9940	2.7	0.0030	0.9991	3.0

[a] Caffeic acid in n-hexane. [b] n = 3, 25 μg ml^{-1}. [c] n = 3, 60 μg ml^{-1}.

since the extraction process takes place through the two interphases corresponding to both ends of the organic zone.

3.4. Features of the method

The calibration graphs were run under optimum conditions using solutions containing different amounts of caffeic acid in n-hexane. Six calibration graphs were obtained using the maximum signal collected after a different number of cycles (from 10 to 20). All show two linear segments: from 15 to 30 μg ml^{-1} and from 35 to 90 μg ml^{-1}. The slope of the linear segment, the regression coefficient and precision (expressed as the relative standard deviation, r.s.d.) for each linear segment and number of cycles are listed in Table 1. The study was carried out only from 10 to 20 cycles both because of the lack of reproducibility for less than 10 cycles and the small further increase in the signal and the long determination time for more than 20 cycles. As can be seen from Table 1, the sensitivity of the method increased with the number of cycles, the increase being greater for the lower linear range. The 3σ detection limit is 10 μg ml^{-1} caffeic acid, lower than that obtained with the previously reported [14] flow-injection liquid–liquid extraction system, although a flow cell with longer optical path was used (10.0 mm vs. 1.5 mm). The r.s.d. decreased with an increasing number of cycles. The sample throughput also depended on the number of cycles programmed (11 samples h^{-1} for $n = 10$ and 8 samples h^{-1} for $n = 20$).

Table 2
Determination of polyphenols in olive oil samples [a]

Sample	Conventional method [b]	Sensor method ± s.d. [c]
1	281,272	270 ± 2
2	186	187 ± 1
3	101, 110	110 ± 1
4	91	95 ± 1
5	328	330 ± 1
6	138	135 ± 1
7	282	290 ± 1
8	272	268 ± 1

[a] Expressed as μg ml^{-1} caffeic acid. [b] [15]. [c] $n = 3$.

3.5. Application of the method

The performance of the method was tested by applying it to the determination of polyphenols in eight different olive oils. The samples diluted (1:5) in hexane were injected in triplicate into the flow-reversal system. The results were compared with those obtained by the method proposed by Vázquez Roncero et al. [15] based on the same chemical reaction. The results obtained by both methods are listed in Table 2. As can be seen, excellent agreement between the two sets of results was found.

4. Conclusions

The proposed method for the determination of polyphenols in olive oil makes use of a very simple system requiring only one (the extraction coil) of the conventional units for liquid–liquid extraction used in continuous configurations and an on-line coupled flow-through sensor that integrates the reaction, retention and detection. The combined system allowed the continuous monitoring of the extraction step. The in situ concentration of the reaction product at the detection improved the detection limit with respect to the flow-injection method that does not use a sensor [14]. Moreover, the method features a substantial advantage over the manual [15] and flow-injection [14] counterparts, viz. it uses less reagent since the reagent was permanently immobilized on the support packed in the flow cell and is safely regenerated after each extraction.

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