Simultaneous Determination of Three Antioxidants in Foods and Cosmetics by Flow Injection Coupled to an Ultra-short Monolithic Column

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Abstract

The combination of an ultra-short C18 monolithic column (5 mm long) with flow injection analysis results in a versatile and efficient system that has been used for the determination of three antioxidants [propylgallate (PG), butylhydroxyanisole (BA), and butylhydroxytoluene (BT)]. Due to the wide variety of polarities of the analytes, two different carriers (carrier A: methanol–water 42% and carrier B: methanol–water 70%) were able to separate the analytes in only 85 s. The applicable concentration range, the detection, and the relative standard deviation (n = 10) were: for PG, from 2.77 to 300 µg/mL, 0.84 µg/mL, 2.84%; for BA, between 1.51 and 300 µg/mL, 0.46 µg/mL, and 2.70%; and for BT, between 1.65 and 100 µg/mL, 0.55 µg/mL, and 2.22%, respectively. The method was applied and validated satisfactorily for the determination of PG, BA, and BT in food and cosmetic samples.

Introduction

Although the inclusion of separation techniques in flow injection analysis (FIA), such as extraction, ion-exchange, or adsorption seeks to isolate the analyte from interfering substances (1), it has also been used for the resolution of mixtures. The resolution of mixtures of analytes based on this system has been accomplished in different ways: (*i*) separation using an on-line minicolumn placed just before the flow cell along with the transient retention of analytes in the solid phase filling this flow cell. The solid phases, minicolumn, and flow cell can be the same or different (2–4); (*ii*) use of a minicolumn to retain one analyte along with the retention of this analyte once it is eluted in the solid phase filling the flow cell, while the second is measured when it flows among the interstitial solution through the particles (5,6); (*iii*) the use of the differences in the transient retention of both analytes in the flow cell. The transient retention of one analyte in the upper part of the flow cell, away from the measuring area, makes it possible to measure the one that is less retained and thus its resolution (7,8); (*iv*) use of a chemometric approach without separation of the analytes prior to the detection step (9,10).

Thus, the use of short columns, as the previously described, filled with large particulate solids to minimize the pressure drops results in additional selectivity but with low resolution power, typically no more than two analytes. A good alternative is monolithic materials. These are continuous pieces of macroporous material (as much organic polymers as silica-based) obtained by polymerization. The resulting high permeable monolith exhibits a three-dimensional network with a high surface area with mesopores (2–50 nm) for retention and macropores [i.e., larger channels (0.5–2 µm diameter)] for through-flow and its functionality can be tailored according their use (11). These relatively recent materials (12,13) have found a wide application in separation sciences due to their high permeability and the reduced back pressure restriction they show, such as in chromatography including gas chromatography (GC) and liquid chromatography (LC) both in high-performance LC (13) and low pressure chromatography (14,15) as well as CEC (16) both in conventional instruments and in microfluidic devices (17).

The combination of low-pressure chromatographic separations with flow techniques can be considered as a low-cost methodology to achieve the required selectivity for analysis (18). In this way, the combination of flow injection analysis (FIA), sequential injection analysis (SIA), or multisyringe flow injection analysis (MSFIA) with chromatography using monolithic columns results in the so-called hybrid FIA/HPLC (19,20), sequential injection chromatography (SIC) (21), or multisyringe liquid chromatography (MSC) (18), extending the possibilities of flow techniques.

The main goal of this study was to develop a monolithic column chromatography procedure using an FIA manifold applied to the analysis of three common antioxidants, namely butylated hydroxytoluene (3,5-di-tert-butyl-4-hydroxytoluene;

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E-321) (BT), butylated hydroxyanisole (2- and 3-tert-butyl-4-hydroxyanisole; E-320) (BA), and *n*-propylgallate (3,4,5-trihydroxybenzoic acid *n*-propyl ester; E-310) (PG), which are frequently used in foods and cosmetics to prevent oxidation, using an FIA manifold coupled with a monolithic column. The second goal of this work was the characterization and validation of the procedure and its application to different types of real samples. This paper forms part of a study on flow injection chromatography using different types of monoliths as a separative element (22), as is the case of a previous report on additives mixture analysis in cosmetics and foods (23).

Different approaches have been used to determine antioxidant mixtures in foods and cosmetics (24). HPLC using UV (25,26) or electrochemical detection (27,28) is the technique of choice for those compounds, although EC also offers good results (29,30). The use of derivatization reactions makes it possible to analyze antioxidants by GC (31) and GC–mass spectrometry (32). Voltammetric methods have also been employed for phenolic antioxidant mixtures in connection with chemometric techniques (33–35). Kinetic methodology also offers a way to determine synthetic antioxidant mixtures in foods and cosmetics (36). Flow injection analysis with electrochemical detection has been used for BHA–PG mixtures in foods (28). Additionally, we have studied the resolution of binary antioxidants mixtures using FIA schemes using flow cells filled with solid supports (7,8), which results in procedures with good analytical characteristics but with low resolution capacity.

The chromatographic approach used in this paper to determine common antioxidant mixtures was based on the use of ultra-short monolithic columns (5 mm) that maked it possible to develop a simple methodology for the analysis of antioxidants that did not need derivatization reactions or complicated systems.

Experimental

All chemicals used were of analytical-reagent grade. Aqueous solutions were prepared using reverse-osmosis type quality water produced by a Milli-RO 12 plus Milli-Q purification system (Millipore, Bedford, MA).

Butylated hidroxyanisole (BA) (1000.0 mg/L) (Fluka, Madrid, Spain, 98.8 %), *n*-propylgallate (PG) (1000.0 mg/L) (Sigma-Aldrich Química S.A., Madrid, Spain, ≥ 98%), and butylated hidroxytoluene (BT) (1000.0 mg/L) (Fluka, 99%) stock solutions were prepared by the exact weighing of each compound and dissolution in methanol (Lab-Scan, Dublin, Ireland, 99.9% v/v HPLC grade). Those solutions were spectrophotometrically stable when protected from light and stored in a refrigerator at 5°C for at least 2 months. Working solutions were prepared by appropriate dilutions with water while maintaining 42% (v/v) methanol in all instances.

The carrier solutions were methanol (HPLC grade Lab-Scan)–water mixture and acetonitrile (Panreac Quimica S.A., Barcelona, Spain, HPLC-gradient grade)–water mixtures at different ratios. All the solutions were filtered through 0.45-µm GH Polypro hydrophilic polypropylene membrane filters (Pall Corporation, New York, NY) prior to use.

Conventional HPLC instrumentation consisted of a Hewlett Packard 1100 series liquid chromatograph (Agilent Technologies) provided with a C8 Zorbax column and including quaternary pump, solvent degasser system, autosampler and DAD detector.

Flow injection analysis components included a peristaltic pump (Gilson Minipuls-2, Villiers-le-Bel, France) with Tygon peristaltic pump tubing (Saint-Gobain Performance Plastics, Charny, France) working at a constant flow-rate, two variable volume teflon rotary valves (Rheodyne 5041, Rheodyne, Cotati, CA) controlled electromechanically by a homemade method, PTFE tubing (Omnifit, Cambridge, England) (0.8 mm i.d. and 1.6 mm o.d.) and various end-fittings and connectors of different diameters (Omnifit also). The absorption measurements were made with a Hewlett Packard HP-8453 diode array spectrophotometer (Nortwalk, CT) equipped with a Hellma 138-QS flow glass cell with 1-mm light path. The monolithic minicolumn was Chromolith guard cartridges RP-18 endcapped (5 mm × 4.6 mm i.d.) with cartridge holder (Merck, Darmstadt, Germany) and was included in the flow line, before the flow cell, which was placed in the cell compartment of the spectrophotometer. Data was acquired with the UV visible Chemstation software package supplied by HP.

Procedures

Basic Procedure

The sample solution (50 µL) containing a mixture of PG, BA, and BT in concentrations comprising between 2.77 to 300 µg/mL for PG, between 1.51 to 300 µg/mL for BA, and between 1.65 to 100 µg/mL for BT was inserted into the flow analysis system (through an injection valve), with the same composition as the carrier. The carrier stream (42% MeOH–H_2O, v/v) at a flow-rate of 2.4 mL/min was changed from 42% to 70% methanol 30 s after the injection valve was opened through the selection valve. When the sample plug reached the monolithic minicolumn, the analytes were separated according to their polarity. The first analyte to be detected was PG (retention time 15 s at 273 nm), followed by BA (47 s at 288 nm), and BT (74 s at 279 nm). Then the flow system was conditioned with the carrier A for 85 s. The relationship between the concentration and peak area was established by conventional calibration with external standards.

Reference procedure

As reference method, an adaptation of the HPLC–diode array detection (DAD) method proposed by Perrin and Meyer (26) was used. Five micrometer C_8 silica in a 150 × 4.6 mm column was used as a stationary phase. As a mobile phase, a gradient ranging from 72% of carrier A (water at pH 3.0 acidified with phosphoric acid 1% v/v) and 28% of carrier B (methanol–acetonitrile, 1/1) to 10% of carrier A and 90% of carrier B at a constant flow rate of 1.0 mL/min was used. The chromatograms were acquired at a wavelength of 273 nm for PG, 288 nm for BA, and 279 nm for BT. In order to obtain the calibration functions, 5 different concentration levels and 3 replicates of each one of the standards were analyzed using the peak area as the analytical parameter.

Treatment of samples

Of the different extracting procedures proposed in the literature for the extraction of antioxidants in foods and cosmetic commercial products, we selected that indicated by Perrin and Meyer (26) with minor modifications described in this section.

The proposed procedure was applied to the antioxidant analysis in foods (chicken bouillon cubes, dehydrated soups, gum) and in cosmetics (body oil). For the analysis of body oil, an adequate amount (typically 25 g) was diluted with 100 mL of hexane. The solution was extracted with 20 mL of methanol 70% (v/v) three times for 10 min each. The three combined hydroalcoholic extracts were filtered through a Millipore filter of 0.25 µm and were finally levelled to 100 mL. The Basic procedure was then applied (see "Basic procedure" section).

For the analysis of gum, an adequate amount (typically 5 g) was weighed and finely cut in order to improve the contact surface with the solvent, and treated with magnetic stirring with 25 mL of methanol three times for 10 min each. Then, the combined extracts were centrifuged at 2500 rpm for 15 min, rota-evaporated, and re-solved with 25 mL of methanol. Then the "Basic procedure" was applied.

For the analysis of bouillons and dehydrated soups, an adequate amount (typically 5 g) was weighed, crushed, homogenised, and extracted with 25 mL of hexane–2-propanol (1/1) three times for 10 min each. The combined extracts were centrifuged at 2500 rpm for 15 min, and re-extracted another three times with 25 mL of methanol each. The combined methanolic extracts were rota-evaporated and diluted up to 10 mL with methanol. The "Basic procedure" was then applied.

Results and Discussion

Variables

We have previously worked with different FIA systems coupled with minicolumns packed with large particulate solids (4,37), but these systems have two disadvantages: high back-pressure and low separative power. An increase in the separation power through the increase in the column length can lead to an increase in the pressure drop across the columns, in such a way that the use of peristaltic pumps is excluded. The main aim of this study is to develop a chromatographic procedure based on short silica-based monolithic columns for the separation of organic compounds using an FIA manifold. For the monolithic separative minicolumn, we selected the Chromolith commercial guard cartridges (C_{18} reverse phase silica 5 mm in length and 4.6 mm diameter). Working with this ultra-short column, it was possible to solve the usual problem using low-pressure pumps, such as peristaltic pumps (14) for separations on monolithic columns. In this case, the system back-pressure was reduced using tubing with 0.8 mm i.d., and the maximum flow rate attained was 2.4 mL/min.

Eluent conditions

With reverse phase materials, the main variable that influences the elution order is the composition of the carrier used, which is usually composed of a buffer to adjust the pH and a percentage of organic solvent to adjust the polarity. The elution profile of the analytes proved to be independent of the pH of the eluent in the range of 2–6. We tested different hydro-organic mixtures as carriers, obtaining the best results with ACN–water and MeOH–water mixtures.

Figure 1 shows the retention behavior with carrier composition for both mixtures. In all cases, the values plotted are the

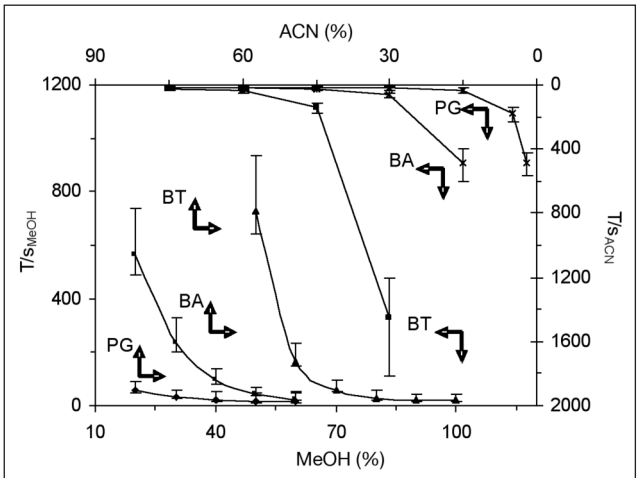

Figure 1. Variation of the retention time vs. % MeOH. Conditions: carrier: variable methanol percentage. Standards: 50 µg/mL of all antioxidants. Flow rate: 1.5 mL/min. Loop volume: 500 µL. Arrows indicates the axis. Error bars indicate for each analyte the bandwidth.

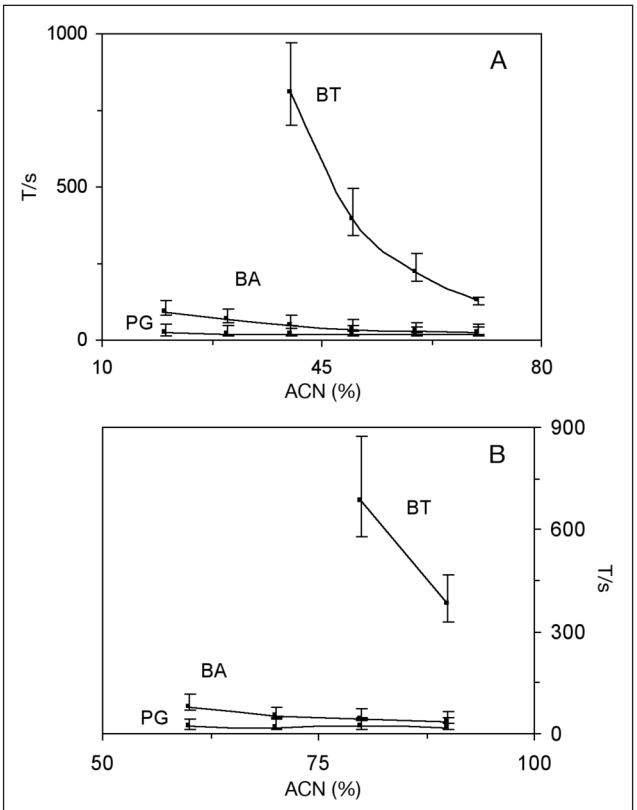

Figure 2. Retention time with ternary carriers ACN–MeOH–water: At 10% MeOH constant (A); At 30% MeOH constant (B). Conditions: 50 µg/mL of all antioxidants. Flow rate: 1.5 mL/min. Loop volume: 500 µL. Error bars indicate for each analyte the bandwidth.

retention times and are the average of three replicates. The bars represent the bandwidth at the base of the peak (i.e., the time passed between the beginning of the peak and the return to the baseline). The standards were diluted with the same hydro-organic percentage as the carrier. The behaviour of the analytes followed their order according to their polarity (PG > BA > BT). However, their large differences in polarity make it impossible to obtain a good resolution within a reasonable analysis time.

The use of ACN–MeOH–H_2O ternary mixtures was studied by settling the percentage of one component and modifying the two others, obtaining the results shown in Figure 2. From prior results we adjusted the MeOH percentage to a constant 30% and studied the behaviour of the analytes in different carriers ranging between 10% and 70% ACN. The antioxidant standards were prepared with the same carrier composition each time and only when the water percentage was higher than 60% did the analytes begin to precipitate. Figure 2A shows that with an ACN percentage of 20%, the resolution between PG and BA decreases below 1.5, thus, those peaks overlap. In these conditions, with an ACN percentage lower than 20%, the analysis time is greater than 1000 s and an increase in the constant MeOH percentage up to 40% results in shorter retention times but greater overlapping.

Additionally, the use of a constant 10% of MeOH (Figure 2B) was studied up to 40% water due to the precipitation of analytes. In this case, percentages of ACN higher than 70% decreased the resolution between PG and BA to below 1.5, with the analysis time in this specific case (70% ACN) being 900 s. As a conclusion, it is not possible separate the three antioxidants by using the cited ternary carriers with good resolution and short analysis time.

To reduce the analysis time, we changed the carrier composition during the FIA analysis, although not in a continuous way (gradient elution type) but by changing from one carrier to another with less polarity through a selection valve. According to the results of the study of binary mixture carriers (Figure 1), the two most polar antioxidants (PG and BA) can be separated with adequate resolution using mixtures of MeOH–water up to 45% MeOH. We selected 42% MeOH as carrier A because it is the highest percentage that permits a good resolution between PG and BA. The carrier B composition needed for the elution of the least polar analyte (BT) was another MeOH–water mixture in order to minimize the baseline alteration due to the change of the carrier in the analysis run. The MeOH percentage and the time of change (CT) of the carrier B were two interrelated parameters. Figure 3 shows the retention time for all three analytes with the methanol percentage at 80 s changing time from carrier A to carrier B, because this time is sufficient to remove BA from the column.

At a first glance, it seems that a carrier B with 80% methanol is the best option, but the carrier change produces a baseline distortion, similar to a chromatographic ghost peak. This dis-

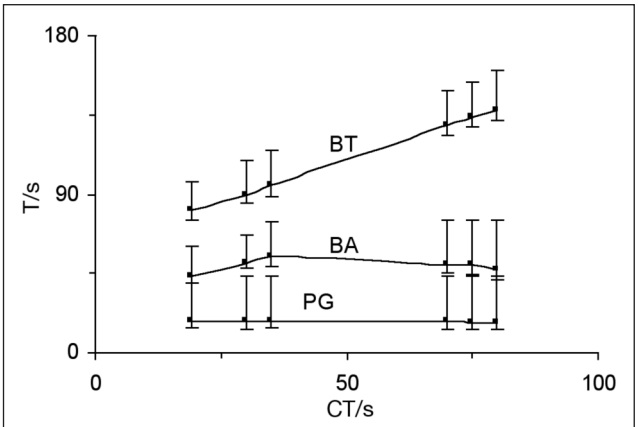

Figure 4. Dependence on the retention time of the change time (CT) from carrier A to carrier B. Conditions: carrier A, 42% methanol–water; carrier B, 70% of methanol–water; Change time, variable; Standards, 50 µg/mL of all antioxidants; Flow rate, 1.5 mL/min; Loop volume, 500 µL. Error bars indicate for each analyte the bandwidth.

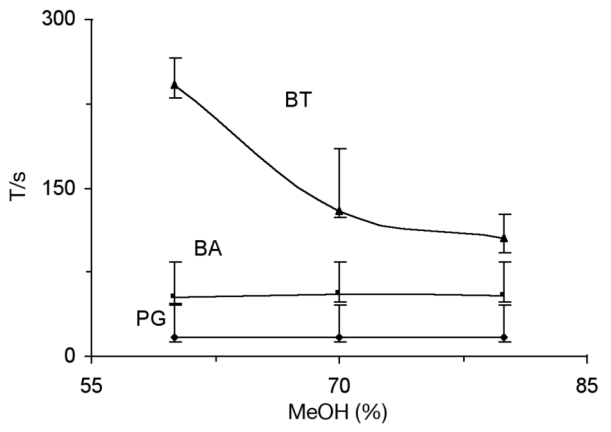

Figure 3. Dependence on the retention time of the percentage of carrier B at constant change time 80 s. Conditions: carrier A, 42% methanol–water; carrier B, variable, from 60% to 80% of methanol–water; Change time, 80 s; Standards, 50 µg/mL of all antioxidants; Flow rate, 1.5 mL/min; Loop volume, 500 µL. Error bars indicate for each analyte the bandwidth.

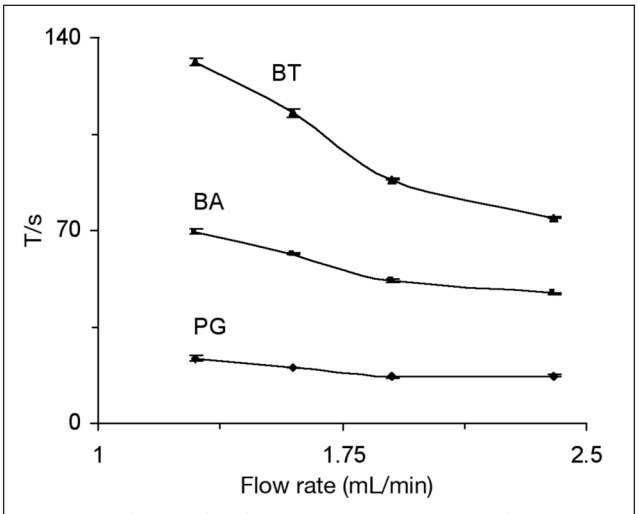

Figure 5. Influence of the flow-rate on the retention time of the antioxidants: Conditions, carrier A, 42% methanol–water; carrier B, 70% of methanol–water; Change time, 30 s; Standards, 50 µg/mL of all antioxidants; Flow rate, variable; Loop volume, 500 µL.

tortion overlaps strongly with the BT signal, and for this reason, we chose 70% methanol–water as the optimum carrier B composition.

The effect of the change time from carrier A to carrier B was studied between 19 and 80 s (Figure 4). With short CT values, the baseline modification could affect BA or even PG signals. On the contrary, large CT values increased the analysis time. We selected 30 s as working CT because the baseline alteration appears just after the BA signal and before, as can be expected, the BT signal.

The FIA variables are flow rate and loop volume. The flow rate was varied between 1.3 and 2.4 mL/min using the same concentration of analytes because higher flow rates are difficult to achieve. We did not use lower flow rates because our main objective was to obtain the quickest possible method. Because of the inclusion of the monolithic column in the flow line, the back pressure increases, but the maximum flow rate reachable with and without column is practically the same. As could be expected, the retention time decreases when the flow rate increases (Figure 5). Additionally, the area decreases as could be expected (16.5% for BT, 16.2% for BA, and 15.8% for PG), the peak height is scarcely modified (+ 6.5% for BT, –6.4% for BA, and +1.1% for PG), the bandwidth at the middle of the peak decreases (13.8% for BT, 22.9% for BA, and 11.0 for PG) and the asymmetry factor remains practically constant. Thus, a flow-rate of 2.4 mL/min was selected because the analysis time is the shortest (85 s).

An increase in the sample volume, studied between 10 and 250 µL, means an increase, as could be expected, in the analytical signal for all the analytes as a result of the larger amount of analytes in the flow system. The retention time suffers a slight increase when loop volume increases (3.1% for BT, 5.0% for BA, and 1.2% for PG). The analytical signal in the area increases 8.3% for BT, 14.9% for BA, and 20.1% for PG; and in FIA, the height increases 11.6% for BT, 13.3% for BA, and 18.2% for PG. The bandwidth is practically not affected (it increases 1.9 % for BT, 8.1 % for BA and 3.6 % for PG), and the asymmetry factor increases 14.8% for BT, 29.7% for BA, and 4.7% for PG. Thus, we selected 50 µL as the optimum sample volume, because it is the largest volume (and, thus, represents the biggest analytical signal) that does not reduce the asymmetry of the peaks with a retention time that is not very high. Working at 50 mg/L for the three antioxidants, the within-day precision of the retention time was PG 0.48%, BA 1.27%, and BT 0.08%, and the day-to-day precision was PG 0.45%, BA 1.57%, and BT 1.50%.

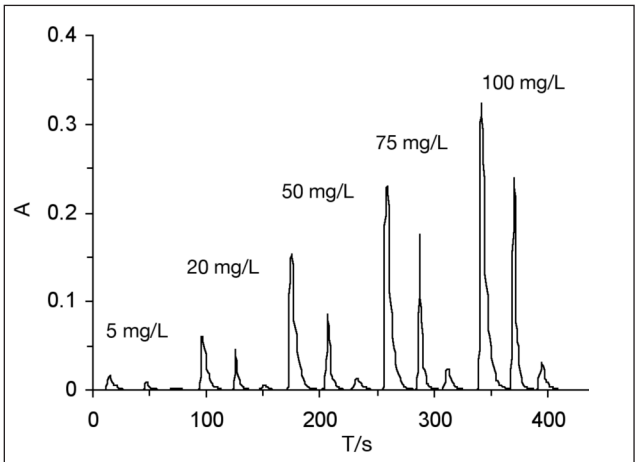

Figure 6. Determination of antioxidants at 5 concentration levels for PG, BA, and BT in the conditions given in the "Basic procedure" section.

Analytical features

The area of the FIA peak was used as the analytical signal because it shows better analytical features than height (Table I). For PG, the calibration graph was linear from 2.77 to 300 µg/mL, for BHA from 1.51 to 300 µg/mL, and for BHT from 1.65 to 100 µg/mL when analytical signals were collected at the wavelength of the maxima for each of them (Figure 6). The adjustment of those analytical data was carried out by linear regression, with the lack-of-fit test applied to test the linearity (three replicates of each standard and ten standards for each calibration graphs). The standard deviation of the background signal measured for the blank, which is necessary for the estimation of the IUPAC detection limit (K = 3) and the quantification limit (K = 10)(38), was taken as the average of 10 determinations and noted as RSD units. Table I shows the analytical parameters for the proposed procedure. Because the analytical parameters were obtained with a 1-mm flow cell, the use of the longer path length results in better analytical figures. The sampling frequency is 22 h. The proposed method shows satisfactory results for the quantification of each antioxidant in the presence of different ratios of the co-existing antioxidant. Eleven antioxidant mixtures with ratios in the range 1:100 to 100:1 were added at levels of 3 to 300 mg/L for PG and at levels of 1.5 to 300 mg/L for BA and BT. Recovery of antioxidants was in the range of 100.3% to 105.3% for PG, 97.3% to 101.6% for BA, and of 98.5% to 102.5% for BT, with mean recovery of 102.9% for PG, 99.8% for BA, and 100.5% for BT.

Table I. Analytical Parameters for the Mixtures BA, BT, and PG*

	Area		
Parameter	PG 273 nm	BA 288 nm	BT 279 nm
B (mL/µg)	20.56	7.91	7.17
S_b	0.17	0.05	0.07
a	–58.79	–9.28	1.69
S_a	16.94	6.15	3.24
R2 (%)	99.78	99.84	99.81
PL (%)	42.20	49.74	15.98
LDR (mL/µg)	2.77 to 300	1.51 to 300	1.65 to 100
LOD (mL/µg)	0.84	0.46	0.55
LOQ (mL/µg)	2.80	1.53	1.83
RSD (%)	2.84	2.70	2.22

* *b* = Slope; S_b = standard deviation of slope; *a* = intercept; S_a = standard deviation of intercept; R = correlation coefficient; PL = probability level of lack-of-fit test; LDR = Linear dynamic range; LOD = limit of detection; LOQ = limit of quantification; RSD = relative standard deviation.

Applications

The proposed flow-through method was applied to the determination of antioxidants in foods (chicken bouillons, dehydrated soups, and gum) and cosmetics (body oil), obtained from different supermarkets in the town of Granada (Spain). These commercial products contain two antioxidants (PG and BT in the body oil; BA and BT in the chewing gum and dehydrated soup, and PG and BA in bouillon cube). In order to check the method, we powered one of these (bouillon cubes) with the antioxidant that it does not contain (BT).

We demonstrated that the extraction step used gives quantitative results through the addition of known amounts of each antioxidant. The recovery experiments produced results ranging between 95.4% and 98.2% for PG, between 96.0% and 98.4% for BA, and between 95.18% and 96.5% for BT (using area). Table II shows the results obtained using the proposed procedure with an HPLC procedure (26) used as a reference. Table II includes the mean values from 3 determinations of each sample, standard deviations of these measurements, and the probability value (Pval) of the test used for the comparison of the results (P-value) obtained for both methods. As can be seen, the results obtained for both methods are statistically similar.

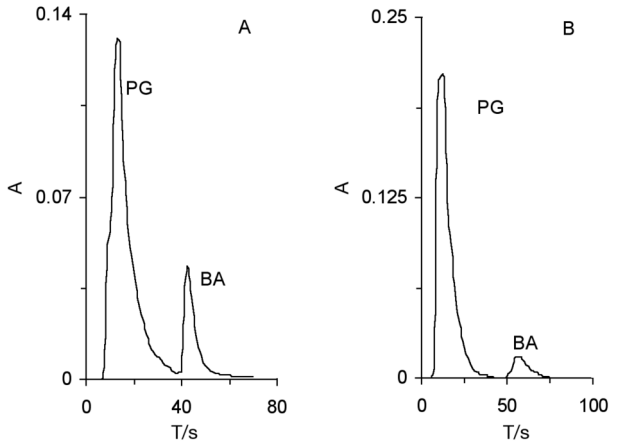

Figure 7. Determination of antioxidants using ultra-short monolithic column in bouillon cubes (A) and dehydrated soup (B).

The proposed chromatographic method shows several advantages against the usual HPLC procedure; it uses very simple, fast instrumentation that is cheaper and has similar precision. The disadvantage is that the other procedure has a better resolution than our procedure, as expected with HPLC, although our method is sufficient for determination.

Conclusion

The incorporation of an ultra-short monolith column (5 mm) into FIA results in an interesting approach for implementing multi-analyte chromatographic determinations using simple, versatile, and low-cost methodology. The proposed method makes it possible to determine three common antioxidants, PG, BA, and BT at µg/mL level in food and cosmetics products without any prior derivatization reaction, and it may be employed in routine analysis.

The FIA method presented here has several advantages over the usual chromatographic methodologies. The advantages

Table II. Results Obtained by Proposed and Reference Procedures in Foods and Cosmetic Samples

	PG (mg/kg); S (%)			BA (mg/kg); S (%)			BT (mg/kg); S (%)		
Sample	Proposed Method	Reference Method	P-value (%)	Reference Method	Proposed Method	P-value (%)	Proposed Method	Reference Method	P-value (%)
Body oil	53.7; 0.22	54.1; 0.50	12.19	–	–	–	505.0; 0.67	504.60; 0.04	83.26
Chewing gum	–	–	–	24.2; 0.37	24; 1.43	35.60	139; 1.70	139; 1.30	78.43
Dehydrated soup 1	273.3; 0.42	273.9; 0.55	65.66	143; 2.68	143; 2.52	96.80	–	–	–
Dehydrated soup 2	263; 1.55	266.5; 0.35	18.53	257; 3.35	258; 2.51	80.66	–	–	–
One-minute soup	11; 3.30	11; 3.01	89.20	27; 2.59	27; 2.67	97.37	–	–	–
Bouillon cube	36; 2.70	36; 4.64	79.37	24; 3.12	24; 1.24	73.67	–	–	–
Bouillon cube spiked with BT*	36; 1.30	35.9; 0.23	21.45	24; 1.80	24; 1.22	51.12	197; 1.66	194.1; 0.51	10.22

* Spiked with 200 mg/kg. For each sample the mean from 3 determinations and the standard deviation is included. Pval is the probability value of the test used for the comparison of the results.

concern the possibility of performing the analysis using low-cost instrumentation with the inclusion of different sample handling operations, and additionally with short analysis time, low consumption of solvents, and low cost per analysis. The drawbacks of this methodology are related to its limited capacity for separation due to the column length.

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