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Determination of catechins by means of extraction with pressurized liquids☆

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Abstract

The technique of extraction with pressurized liquids is applied for extraction of catechin and epicatechin in tea leaves and in grape seeds. The extracts obtained are then analyzed by HPLC. A comparison has been made of the recoveries obtained employing extraction by magnetic stirring, ultrasound-assisted extraction, and extraction with pressurized liquids. In the three extraction systems, four different pure solvents were utilized, namely water, methanol, ethanol, and ethyl acetate. Methanol produced the best results. For this comparison, an initial step was to check the stability of catechins during the process of extraction using pressurized liquids at high temperature (100–200 °C). It has been confirmed that recoveries of these two compounds begin to fall, to below 95%, at 130 °C and above. Pressurized liquid extraction using methanol as solvent, produces results, in terms of recovery of catechin and of epicatechin, notably higher than any of the other conditions of extraction tested. The duration of the extraction cycle was also evaluated and re-extractions were performed to ensure the full recovery from the samples. It was found that, for catechin, the R.S.D. of the method is 3.21%, and for epicatechin the R.S.D. was 2.96% ($n = 5$). The final optimized pressurized liquid extraction method allows for the determination of catechin and epicatechin in diverse types of samples with a rapid (10 min) and reproducible method.

Keywords: Extraction methods; Pressurized liquid chromatography; Tea; Grapes; Food analysis; Catechins; Antioxidants; Flavanols

1. Introduction

Catechin and epicatechin are two flavanols of the catechin family that are present in many foods of plant origin. The considerable interest in these compounds is due to their wide range of beneficial effects for human health [1–8] (see [8] for a review).

These compounds can be ingested from plant foods and drinks derived from these. In human beings, the absorption of catechin in the blood and the formation of derivatives after the ingestion of red wine have been proven [9].

From the analytical perspective, the determination of these two catechins in liquid samples has been widely developed, both by means of HPLC [10] and even by GC [9]. In solid foods, however, extraction processes are necessary, and in many instances, these require long times for the maceration of the sample with organic solvents [11]. For this reason, some researchers have resorted to extraction with fluids in supercritical state [12,13] and to the application of solvents above their boiling point [14,15]. In recent years, numerous methodologies for the extraction of compounds of relatively high polarity employing pressurized liquid extraction (PLE) in place of supercritical fluid extraction (SFE) have been developed [16] as a consequence of the low polarity of the supercritical fluids available. This paper presents the results obtained in the application of PLE for the extraction of catechins from samples of both grape seeds and tea leaves.

In the HPLC analysis of the extracts obtained, a diode array detector and a fluorescence detector have been employed in-line, taking advantage of the spectroscopic properties of catechins [17] and with the aim of obtaining lower detection limits.

2. Experimental

Ethanol (Panreac, Barcelona, Spain), methanol, ethyl acetate, and acetic acid (Merck, Darmstadt, Germany) used

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were HPLC grade. Water was supplied by a Milli-Q water purifier system from Milipore (Bedford, MA, USA). Catechin and epicatechin standards were obtained from Sigma (St. Louis, MO, USA).

The ultrasound-assisted extractions were carried out in a high-intensity ultrasound probe system of 200 W and 24 kHz (model UP 200S, Dr. Hielscher GmbH, Germany) equipped with a 2 mm microtip. Its ultrasonic vibrations amplitude controller was set to 100% of nominal power.

An ASE-200 extractor (Dionex, Sunnyvale, CA, USA) was used for the pressurized liquid extractions. The extraction cell volume was 11 ml and the collection vial volume was 40 ml. Sea sand (Panreac) has been used as supporting material in the extraction chamber.

The analyses of the extracts were performed by HPLC in a Waters system consisting of an autosampler (717 plus), pump controller (600S), pump (616), a photodiode array detector (996) and a fluorescence detector (474), using a RP-18 column (LiChrospher 100, 250 mm × 3 mm, 5 μm particle size, Merck, Germany) and a gradient of acidified water (2% acetic acid) (solvent A) and methanol–water–acetic acid (90:8:2) (solvent B) at a flow rate of 0.3 ml/min. The gradient was as follows: 0 min, 20% B; 10 min, 25% B; 20 min, 50% B; 21 min, 100% B. The UV absorbance was monitored from 200 to 400 nm. The identification of catechin and epicatechin was made by comparison of retention times with pure standards, as well as by UV-Visible spectra. Fluorescence signal was used for quantification purposes. The fluorescence output signal (excitation wavelength 290 nm, emission wavelength 320 nm) was monitored and integrated using Millennium^{32} Chromatography Manager software (Waters). A stock solution of catechin (100 mg l^{-1}) and epicatechin (100 mg l^{-1}) was prepared in methanol–water (1:1). The stock solution was diluted to give different standard solutions. The resulting calibration curves were $y = 160147x + 4630$ ($R^2 = 0.9999$) for catechin and $y = 135987x + 2532$ ($R^2 = 0.9997$) for epicatechin.

3. Results and discussion

3.1. HPLC method

With the aim of developing a more selective method of determination by HPLC, with a lower limit of detection, the fluorescence excitation spectra and the emission spectra of catechin and epicatechin were analyzed. Both spectra are shown in Fig. 1. Based on these, wavelengths of 290 nm as excitation and of 320 nm as emission have been utilized for the selective detection of catechins; these conditions almost coincide with those described in the bibliography [17]. With these conditions, a chromatogram like that of Fig. 2 is obtained on analyzing extracts of real samples, specifically those corresponding to tea.

The repeatability of the HPLC method of analysis was evaluated employing extracts of samples of tea, and the R.S.D. ($n = 5$) were found to be 0.8 and 0.7%, respectively, for catechin and epicatechin.

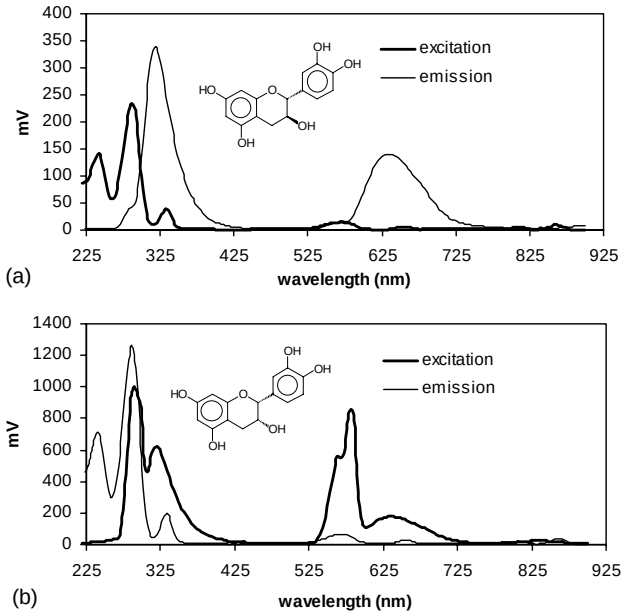

Fig. 1. Fluorescence excitation and emission spectra for catechin (a) and epicatechin (b).

3.2. Comparison of extraction methods

A comparison has been made of the recoveries obtained from grape seeds (0.5 g) employing extraction by magnetic stirring, ultrasound-assisted extraction, and extraction with pressurized liquids. In the three extraction systems, four different pure solvents were utilized, namely water, methanol, ethanol, and ethyl acetate. In the case of stirring-assisted extraction (SAE) and ultrasound-assisted extraction (UAE), these were conducted at two different temperatures, 10 and 60 °C; since some of the extracting solvent present a boiling point slightly above 60 °C. For PLE, 100 °C was employed as extracting temperature, utilizing a pressure of 100 atm to keep the solvents in the liquid state (1 atm = 101 325 Pa).

The results obtained are shown in Table 1. It can be confirmed that, at 60 °C, the best results are obtained employing

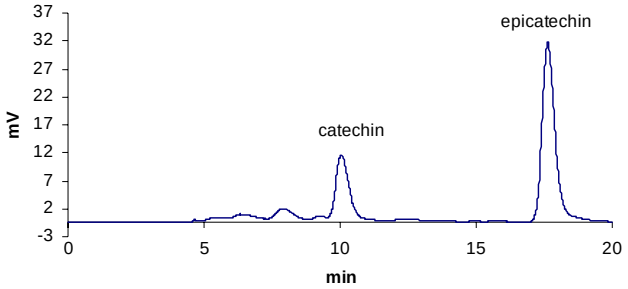

Fig. 2. Chromatogram of an extract obtained by PLE of non-fermented tea leaves. Fluorescence detection conditions: excitation, 290 nm; emission, 320 nm.

Table 1
Recoveries obtained using pressurized liquid extraction (100 °C), ultrasound-assisted extraction and stirring-assisted extraction at 60 and 10 °C for catechin and epicatechin

	Catechin (mg/g)				Epicatechin (mg/g)			
	Water	Methanol	Ethanol	Ethyl acetate	Water	Methanol	Ethanol	Ethyl acetate
PLE	0.13	1.90	0.59	0.15	0.09	0.72	0.25	0.02
UAE-60	0.27	0.93	0.29	0.64	0.24	0.46	0.17	0.23
SAE-60	0.15	0.85	0.30	0.23	0.13	0.44	0.18	0.23
UAE-10	0.00	0.59	0.12	0.35	0.00	0.28	0.06	0.13
SAE-10	0.00	0.59	0.08	0.32	0.00	0.28	0.04	0.13

methanol as the solvent. The recoveries obtained with methanol are appreciably greater than those obtained with any of the other solvents, in both the SAE and UAE systems, and for both catechin and epicatechin. The results obtained in the experiments conducted at 10 °C are similar in respect of the conclusion that methanol is again the best solvent, producing greater recoveries. In this case, it should be emphasized that the four solvents produce recoveries that are between 30 and 50% lower than those obtained when 60 °C is employed as the extraction temperature; especially notable is the case of water since, in the extracts obtained with water at 10 °C, neither catechin nor epicatechin was detected.

Regarding the results obtained employing PLE, again methanol is the solvent that produces the best results, in this case also with notable differences with respect to the other three solvents. It should also be noted that the results of the extracts obtained by PLE are better than those obtained with the other two techniques for methanol and for ethanol, while for water, the best conditions of extraction are UAE at 60 °C, and for ethyl acetate, the recoveries obtained employing PLE are even lower than those obtained using UAE or SAE at 10 °C.

From all this, it can be concluded that the employment of PLE using methanol as solvent, produces results, in terms of recovery of catechin and of epicatechin, notably higher than any of the other conditions of extraction tested for the case of grape seeds. In the case of PLE, the possibility is also presented of employing a wider range of temperatures, even above 100 °C, that could increase the recovery or else diminish the time required to obtain the same recovery.

3.3. Optimization of PLE

Extractions were performed between 100 and 200 °C by PLE in order to determine the optimum temperature for the extraction of catechin and epicatechin from grape seeds. The results of the recovery of both compounds obtained are presented in Fig. 3. The same figure presents the results obtained employing times of extraction of 5 and 10 min. For both times, the behavior is similar, the extraction of catechins is increased notably from 100 °C up to the range of 160–180 °C and then diminishes, also drastically, up to the maximum temperature tested, 200 °C. This behavior can be attributed to the superposition of two different effects due to the increase of the temperature. The first of these effects is the greater facility of extraction at higher temperatures, due to the weakening of the bonds between the catechins and the matrix. The second effect, in this case of contrary consequences, is the degradation of these compounds at high temperatures, even in an atmosphere of nitrogen, as was applied during the PLE.

It is also striking that, in the extractions that were performed for only 5 min, the recoveries obtained at temperatures between 150 and 180 °C are appreciably higher than those obtained when the duration of the extraction is 10 min. The only explanation for this finding is that, at those temperatures, the effect of the degradation overrides the effect of increased extraction from the sample. Therefore, the stability of catechin and epicatechin was determined employing a standard solution in 10 min extractions at different temperatures. The results obtained are shown in Fig. 4. It can be confirmed that the recovery is more than 95% with temperatures of extraction of up to 130 °C. Above this temperature, the degradation of both compounds exceeds 5% of

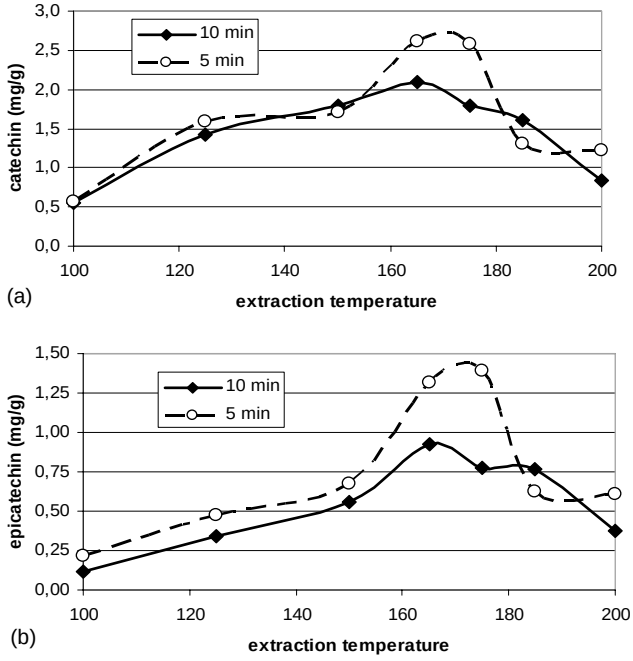

Fig. 3. Recoveries for catechin (a) and epicatechin (b) from grape seeds during the pressurized liquid extraction at different temperatures using two different extraction times: 5 and 10 min.

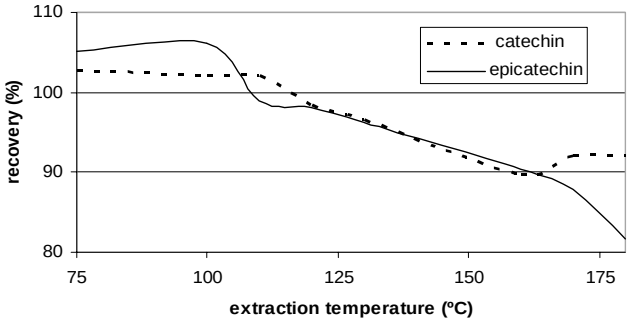

Fig. 4. Recoveries for catechin (a) and epicatechin (b) standards during the pressurized liquid extraction at different temperatures.

the sample submitted to extraction. Hence, the effect of the increased degradation of the catechins during the extraction of 10 min duration at temperatures higher than 130 °C, has been proven. This effect gets stronger, the higher the temperature, reaching the point of compensating fully for the increase in the degree of extraction of the catechins from the real samples. For this reason, the maximum temperature of extraction that can be applied in the PLE system is 130 °C when the extraction duration is 10 min.

3.4. Application to real samples

After the determination of the optimum temperature of extraction, the next step was to apply the PLE method developed, to a wide variety of real samples. In this case, instead of applying an extraction of 10 min, it was decided to apply an extraction consisting of two cycles of 5 min each. The classic SAE and UAE methods, both of 10 min duration, were applied simultaneously, together with an extraction by static maceration of 24 h duration. The samples analyzed were grape seeds and various types of tea, specifically of different degrees of fermentation.

Table 2 shows the recoveries obtained from applying the various extraction methods to grape seeds. As can be observed, the recoveries obtained employing PLE were appreciably higher than those obtained with any of the other three extraction methods. The results are around five times higher than those obtained in the static extraction (SE) during 24 h. The methods based on the magnetic stirring or on the ultrasound-assisted extraction produce substantially lower recoveries.

With reference to tea, the study covered the extraction of three types that differed in the degree of fermentation undergone during their production process. During the fermentation of tea, a process of oxidation of the catechins takes place. This process of fermentation takes place on the surface of the leaf, with the result that the remaining catechins that are not oxidized are located in the less accessible parts of the leaf. Therefore, in principle, the extraction of catechins from fermented teas will present more difficulties than extraction from green tea leaves.

In Table 2, the results obtained in the extraction of green tea (non-fermented tea) are shown. For epicatechin, which is notably the majority catechin, the results of the four extraction methods were very similar, they ranged between 3.16 and 3.45 mg/g. However, for catechin, the results were significantly different, ranging between 0.62 mg/g in the PLE method and 0.23 mg/g in the SAE and UAE methods.

For the tea of medium fermentation (Table 2), the results were similar. The PLE method produces better results for catechin than the other methods. However, for epicatechin, UAE produced a recovery of 2.23 mg/g against 1.86 mg/g obtained by PLE. In this case, if the intention is to apply PLE as the method of extraction, it would be necessary to determine by means of re-extractions the duration of time necessary for the method of extraction, to ensure that the process was quantitative.

In the case of intensively fermented tea, known as black tea, the results are also shown in Table 2. In this case, it can be observed that the most suitable method of extraction is clearly PLE, since for the two compounds it produces recoveries much higher than the other three extraction methods.

From all these results, one can conclude that, in some cases, UAE may produce better results of recovery than PLE at equal periods of extraction time; however, in the majority of occasions, extraction by means of PLE produces greater recoveries than any other method of extraction of catechins.

3.5. Repeatability

To determine the repeatability of the PLE method, extractions were performed on aliquots of the same sample, specifically a sample of green tea leaves. It was found that, for catechin, the R.S.D. of the method is 3.21%, and for epicatechin the R.S.D. was 2.96% ($n = 5$).

Table 2
Recoveries (mg/g) for catechin and epicatechin from grape seeds, non-fermented tea leaves, medium-fermented tea leaves and fermented tea leaves using pressurized liquid extraction (10 min), stirring-assisted extraction (10 min), ultrasound-assisted extraction (10 min) and static extraction (24 h)

	Grape seeds		Non-fermented tea leaves		Medium-fermented tea leaves		Fermented tea leaves	
	Catechin	Epicatechin	Catechin	Epicatechin	Catechin	Epicatechin	Catechin	Epicatechin
PLE (10 min)	1.82	0.65	0.62	3.31	0.57	1.86	0.61	1.03
SAE (10 min)	0.21	0.06	0.23	3.45	0.16	0.81	0.12	0.10
UAE (10 min)	0.23	0.07	0.23	3.36	0.46	2.23	0.22	0.19
SE (24 h)	0.27	0.08	0.30	3.16	0.47	1.97	0.36	0.33

4. Conclusions

The application of PLE enables the determination of catechin and epicatechin in diverse types of samples with rapid (10 min) and reproducible extraction methods. Only in particular instances does UAE present greater recoveries than PLE. The degradation suffered by both compounds at 130 °C turns out to be insignificant, and therefore this temperature can be employed during the extraction of both compounds.

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