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Polyphenols in greenhouse and open-air-grown lettuce

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Abstract

Lactuca sativa L. plants (cv. Audran) developed in greenhouse or in open air, were analysed for their polyphenol compounds (caffeic acid derivatives, quercetin and kaempferol glycosides) to verify whether these two different growing environments affected both the qualitative and quantitative phenol patterns. The lettuce extracts from greenhouse and open-air samples were compared and directly analysed by HPLC/DAD, HPLC/MS and HPTLC. All open-air samples had higher flavonol contents than the greenhouse ones. The applied rapid and sensitive HPTLC method could be routinely employed to determine the leaf flavonol content of a large number of lettuce samples. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: HPLC; HPTLC; Flavonols; Lettuce leaves

1. Introduction

Lactuca sativa L. is an annual herbaceous plant belonging to the Compositae (Asteraceae), one of the largest and most diverse families of flowering plants, comprising one-tenth of all known angiosperm species. Lettuce is native to the Mediterranean area and its cultivation may have started in Egypt as early as 4500 BC, perhaps initially for the edible oil extracted from its seeds. Salad lettuce was popular with the Ancient Greeks and Romans and it arrived in the US during colonial days. Today lettuce is an important crop species and is the most important salad vegetable grown in the US (Davis, Subbarao, & Kurtz, 1997). Lettuce is a minimally processed food-product and its use has continued to increase in salad bars and fast foods because of its long storage life, good quality and its perception as being a healthy food (Ferreres, Gil, Castaner, & Tomas-Barberan, 1997).

The nutrient content of this vegetable includes useful amounts of several important components, such as phenolic antioxidants, vitamins A and C, calcium and iron. In folk medicine, *Lactuca sativa* L. seeds are used in powder form for the treatment of rhinitis, asthma, cough, and pertussis, and its decoction is employed for the treatment of insomnia and as a sedative; moreover, the oil extracted from the seeds has shown some analgesic effect after external application onto the head (Said, Kashef, Mazar, & Salama, 1996).

Several studies of the phenol and polyphenol composition in different cultivars of lettuce have been performed and two main classes of products have been identified: caffeic acid derivatives (Ke & Saltveit, 1988) and flavonols (Hermann, 1976). In particular, monocaffeoyl tartaric acid, chicoric acid (dicaffeoyltartaric acid), 5-caffeoylquinic acid (chlorogenic acid) and 3,5-di-O-caffeoylquinic acid (isochlorogenic acid) are present, and, among flavonols, quercetin 3-O-(6-O-malonylglucoside)-7-O-glucoside, quercetin 3-O-glucuronide, quercetin 3-O-glucoside and quercetin 3-O-(6-O-malonylglucoside) have been reported (Ferreres et al., 1997; Winter & Hermann, 1986).

The flavonoid content of lettuce leaves and other commercial vegetables (onion, celery and tomatoes) was reviewed by Crozier (Croizer et al., 1997), while Bilyk and Sapers (1985) found more quercetin in the outer

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than in the inner leaves. A particularly detailed study of flavonols in lettuce was made by DuPont, Mondin, Williamson, and Price (2000).

The flavonoids are secondary metabolism products, which are widely distributed in plants and seem to play many different roles. As well as biochemical markers, they have an ecological importance in plants: they are involved in protection mechanisms against insects and pathogens, UV light damage (Cooper-Driver & Bhattacharya, 1998; Tattini, Gravano, Pinelli, Mulinacci, & Romani, in press), and in the regulation of plant growth and development. In particular flavonoid biosynthesis is strongly regulated by several environmental stimuli (Romani, Pinelli, Mulinacci, Vincieri, Gravano, & Tattini, 2000) such as excess light, pathogen attacks or wounding (Caldwell, Robberecht, & Flint, 1983; Dixon & Paiva, 1995). The flavonoids act as a barrier for damaging UV radiation owing to their adsorption maxima in the UV region (Cooper-Driver & Bhattacharya, 1998). Some studies have been performed on the influence of solar radiation (UV-A and UV-B) on polyphenol composition of *L. sativa* (cv. New Red Fire) and it was demonstrated that the UV absorbance of leaf extracts at 270, 300 and 330 nm was significantly reduced when UV-B ambient radiation was excluded (Krizek, Britz, & Mirecki, 1998).

Some polyphenol compounds have interesting antioxidant properties (Yamasaki, Sakihama, & Ikehama, 1997) and may protect animal cell metabolism from detrimental effects of reactive oxygen species that occur under severe stress (Husain, Cillard, & Cillard, 1987). They may consequently have an important role in human health (Wang, Xia, Yang, Natschke, & Lee, 1998). Both polyphenol classes have a considerable antioxidant activity (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). In particular, for flavonols, in vitro studies have shown a high antioxidant capacity, mainly based on scavenging of oxygen radicals (Bohm, Boeing, Hempel, Raab, & Kroke, 1997). Currently, the role of naturally-occurring polyphenols, with special emphasis on flavonoids, has been extensively investigated also in animal cell metabolism, in an attempt to relate both in vivo and in vitro biological activity of these secondary metabolites to their chemical structure (Hertog, 1993; Qiong, Balou, Shengrong, Jingwu, Jungai, & Wenjuan, 1999; Sichel, Corsaro, Scalia, Di Bilio, & Bonomo, 1991; Vlietinck, de-Bruyne, Apers, & Pieters, 1998).

The object of this study, which was conducted on *L. sativa* L. plants, that had developed in greenhouse or in open air, was to determine the polyphenolic compounds in order to verify how these two different environments can affect both the qualitative and the quantitative phenol patterns. The findings reported herein confirm the presence of several previously described polyphenols. Moreover, a comparison between HPLC-DAD and HPTLC data was carried out in order to achieve a simple and rapid analysis of the flavonol content of a large number of lettuce extracts.

2. Materials and methods

2.1. Plant material

In this study the outer leaves of lettuce plants (cv. Audran) were analysed. The plants were transplanted on a peat culture substrate 10 days after seed germination and were then divided into two groups. The first group was put outside in open air and the second was left in a polycarbonate greenhouse. The leaves were then collected from the two groups at different dates in May–June 2000 (0, 2, 5, 9, 12, 16 20 and 27 days after transplanting) with three replicates within each sampling. Total integrated photon flux density was monitored for all periods before and after the sampling date using a 1800 LI-COR spectroradiometer (LI-COR Inc., Lincoln, NB, USA). In the greenhouse, maximum PPF (photon photosynthetic flow) was 800 $\mu E\ m^{-2}\ s^{-1}$ with the temperature between 15 and 29 °C; in the open air the maximum PPF was of 1100 $\mu E\ m^{-2}\ s^{-1}$ with temperature between 10 and 20 °C.

2.2. Sample preparation

Leaf lamina were rapidly frozen in liquid nitrogen and stored at −80 °C before proceeding with the analysis. Frozen leaf tissue was then ground in a mortar with a pestle under liquid nitrogen. A quantity of 1 g of fresh tissue was extracted with 4×30 ml of CH_3OH, 80% v/v. The raw methanolic extract was then evaporated to dryness under vacuum (Rotavapor 144 R, Büchi, Switzerland) at room temperature and rinsed with a $CH_3CN/CH_3OH/H_2O$ (pH = 2 by HCOOH) 60:20:20 to a final volume of 5 ml. A sample of 25 μl was analysed by HPLC/DAD and HPLC/MS.

2.3. Experimental procedures

2.3.1. HPLC/DAD analysis

Analyses were conducted using an HP 1090L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (all from Hewlett-Packard, Palo Alto, CA, USA). Polyphenol compounds were separated using a 150×3.0-mm (5 μm) Luna C18 (2) column (Chemtek analytica, Bologna), operating at 26 °C, equipped with a 4 mm 1 ×3.0 mm ID C18 ODS precolumn. A four-step linear solvent gradient system was used, starting from 93% H_2O (adjusted to pH 3.2 by H_3PO_4) up to 75% CH_3CN during a 25-min period at a flow rate of 0.6 ml min^{-1} as detailed in Table 1.

Table 1
The linear solvent gradient system used in HPLC-DAD analysis of polyphenols in lettuce leaves[a]

Time (min)	% Solution H_2O	% Solution CH_3CN
0.1	93.0	7.0
10	86.0	14.0
12	86.0	14.0
16	75.0	25.0
20	25.0	75.0
25	93.0	7.0

[a] Analysis was carried out during a 25-min period at a flow rate of 0.6 ml min^{-1} using a 150×3 mm Luna C18 column (5 μm) operating at 26 °C.

UV-vis spectra were recorded in the 190–450 nm range and the chromatograms were acquired at 240, 280, 330 and 350 nm.

2.3.2. HPLC/MS analysis

HPLC/MS analyses were performed using an HP 1090L liquid chromatograph linked to an HP 1100 MSD Mass spectrometer with an API/Electrospray interface (Hewlett-Packard, Palo Alto, CA, USA). Spectra were registered in negative ion mode applying the same chromatographic condition as previously described. The mass spectrometer operating conditions were: gas temperature 350 °C, nitrogen flow rate 10.0 l min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40 °C and capillary voltage 3500 V. The fragmentor operated at 120 eV.

The identity of polyphenols was ascertained using data from HPLC/DAD and HPLC/MS analyses, by comparison and combination of their retention times, and UV-vis and mass spectra.

2.3.3. Identification and quantification of individual phenolic compounds

Identification of individual polyphenols was carried out using their retention times and both spectroscopic and spectrometric data.

Authentic standards of caffeic acid, 5-caffeoylquinic acid, quercetin 3-O-glucoside (isoquercitrin), were purchased from Extrasynthèse S.A. (Lyon, France). Chicoric acid was extracted and isolated from undeveloped chicory leaves. Quantification of individual polyphenol compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geqslant 0.999$) operating in the range 0–30 μg. Chlorogenic and chicoric acids were calibrated at 330 nm using relative pure standards and, for other caffeic acid derivatives, using caffeic acid as a reference compound.

Finally, an authentic standard was used for the calibration curves of isoquercitrin, obtained at 350 nm.

2.3.4. HPTLC analysis

The extract (2 μl) used for the HPLC analysis was deposited on 10×10 HPTLC layers of SIL C_{18}-50 (Macherey and Nagel) and eluted with $H_2O/CH_3OH/CH_3COOH$ (50:50:6, v/v). After stopping the elution, layers were dipped in a methanol solution containing 1% ethanolamine diphenylborate (NRA reagent). Quantification of spots was carried out with a Shimadzu CS 920 densitometer, scanning at 365 and 440 nm, 24 h after dipping the layers in the diphenylboric acid-(2-aminoethylester). Total flavonoid amount was expressed as isoquercitrin using a three-point regression curve ($r^2 \geqslant 0.998$) operating in the range 0–5 μg.

3. Results and discussion

In Fig. 1, the two chromatographic profiles registered at 350 nm (greenhouse and open air) confirm the presence of two main classes of polyphenols in lettuce leaves: caffeic acid derivatives and flavonols. The HPLC/DAD analysis shows that the open-air samples are richer in flavonoids than the greenhouse ones collected at the same time after transplanting.

In Table 2 the amounts of the main polyphenol compounds in lettuce leaves, namely 5-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, chicoric acid and two caffeic acid derivatives, and the flavonoids quercetin 3-O-glucoside, quercetin 3-O-glucuronide, and quercetin 3-O-(6-O-malonylglucoside) are reported. The sample corresponding to 0 days, which showed the same quali-quantitative profile as the greenhouse sample collected 2 days after transplanting, is not reported in the table. The quercetin glycosides were identified by means of their MS spectra and are the same as those found by Ferreres et al. (1997) in red lettuce, with the exception of quercetin 3-O-(6-O-malonylglucoside) 7-O-glucoside which was not found in this lettuce variety (cv. Audran). All data reported in Table 2 represent an average of three analyses, with the percentage standard deviations ranging from 1 to 3%.

A quantitative variation in polyphenol content could be due, not only to variety, but also to different agronomic conditions, tissue type (red, green or white) and from outer or inner leaves (Bilyk & Sapers, 1985; Crozier et al., 1997; Goupy et al., 1990). Croizer et al. (1997) quantified the conjugated quercetin detected in extracts prior to acid hydrolysis in various lettuce cultivars. His data showed very wide differences in quercetin contents, from 11 to 911 μg g^{-1} fresh weight, whereas kaempferol conjugates are not reported. In the same lettuce variety as our study, according to DuPont et al. (2000), trace amounts of kaempferol 3-O-glucuronide and kaempferol 3-O-glucoside are observed (by HPLC/MS method) in open-air samples. In the present study these compounds were always under 3% of total flavonols.

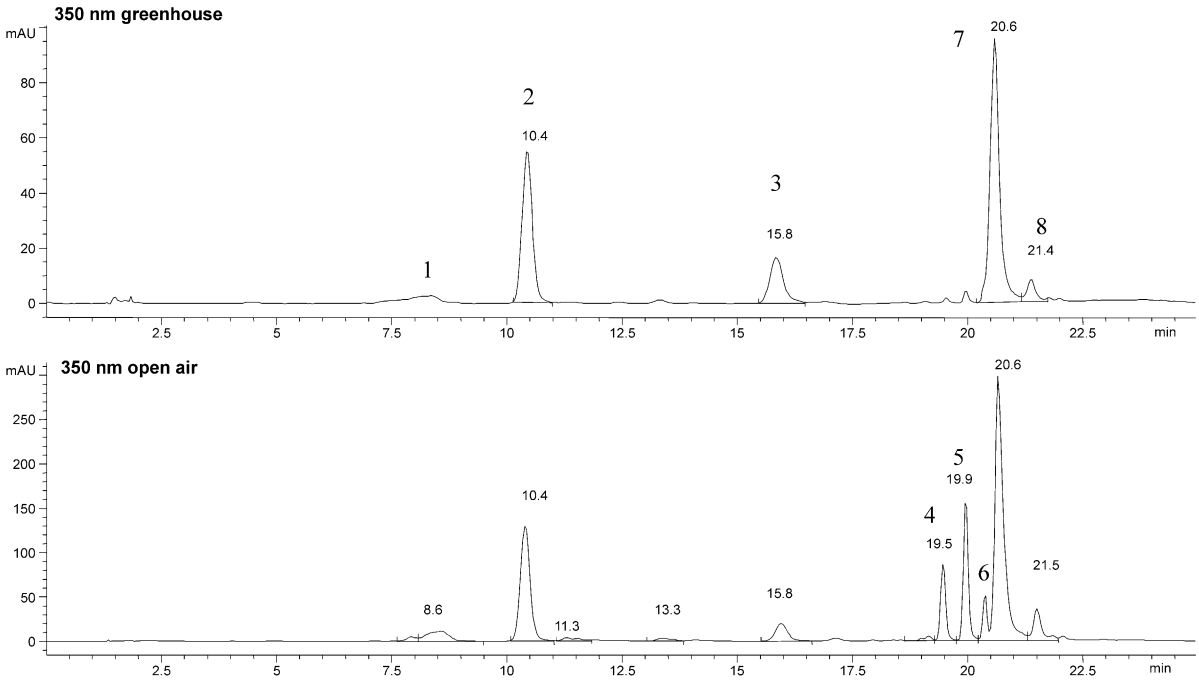

Fig. 1. Chromatographic profile acquired by HPLC-DAD (350 nm) of the methanolic extract from *Lactuca sativa* (cv. Audran) leaves at relative maxima of absorbance of flavonoids in samples growing in greenhouse and open-air, respectively. Eluent was H_2O (pH 3.2 by H_3PO_4)/CH_3CN [4-step linear gradient from 93% H_2O to 75% CH_3CN, see Section 2.3] at a flow rate of 0.6 ml min^{-1} during a 25-min run. Column was Luna C18 (5 μm) maintained at 26 °C. Peaks: 1, caffeic acid derivative; 2, 5-caffeoylquinic acid; 3, isochlorogenic acid; 4, quercetin 3-O-glucoside; 5, quercetin 3-O-glucuronide; 6, quercetin 3-O-(6-malonylglucoside); 7, chicoric acid; 8, caffeic acid derivative.

Among caffeic acid derivatives, chicoric acid shows the highest values, ranging from 0.484 to 1.950 mg g^{-1} of fresh weight, and consititutes more than 55% of total caffeic acid derivatives, except in the samples collected 16 days (2 June) and 27 days (13 June) after transplanting for greenhouse and open-air, respectively.

The total amounts of caffeic, 5-caffeoylquinic, chicoric acid and two additional caffeic acid derivatives, represent more than 93% of the total polyphenols in all of the greenhouse samples and seem to be the only polyphenols present on two collection dates (20 and 27 days after transplanting); these compounds vary from 66 to 94% in the open air samples.

From the reported data, an increasing flavonoid content in the open-air lettuce was observed. The percentage of these compounds in the greenhouse samples was from 0 to 7%, the richest in flavonols being the sample collected 5 days after transplanting. In the open-air

Table 2
Polyphenolic compounds in lettuce leaves evaluated and characterized by HPLC/DAD and HPLC/MS and expressed in mg g^{-1} of fresh weight

	2 days		5 days		9 days		12 days		16 days		20 days		27 days	
	Gh	Oa	Gh	Oa	Gh	Oa	Gh	Oa	Gh	Oa	Gh	Oa	Gh	Oa
Caffeic ac. der.	0.020	0.013	0.074	0.033	0.060	0.048	0.026	0.018	0.019	0.052	0.036	0.041	0.020	0.022
Chlorogenic ac.	0.267	0.544	0.480	1.040	0.338	0.358	0.171	0.342	0.412	0.774	0.176	0.496	0.214	0.572
Isochlorogenic ac.	n. d.	Trace	0.052	0.074	0.070	0.060	0.056	0.080	0.056	0.060	0.037	0.026	0.026	0.034
Chicoric ac.	0.794	1.12	1.95	1.37	1.17	0.962	0.579	1.19	0.484	1.17	0.620	0.765	0.520	0.620
Caffeic ac. der.	0.017	0.016	0.102	Trace	Trace	n. d.	0.010	0.030	Trace	0.012	n. d.	0.012	0.028	0.019
Total phenolic ac.	1.10	1.69	2.66	2.52	1.64	1.43	0.842	1.66	0.971	2.07	0.870	1.34	0.808	1.27
Quercetin 3-O-glucoside	n. d.	0.048	0.013	0.260	n. d.	0.044	n. d.	0.106	n. d.	0.047	n. d.	0.039	n. d.	0.021
Quercetin 3-O-glucuronide	0.031	0.187	0.075	0.380	0.008	0.316	0.003	0.156	0.004	0.143	n. d.	0.101	n. d.	0.033
Quercetin 3-O-(6-malonylglucoside)	0.037	0.295	0.109	0.210	0.008	0.237	0.004	0.161	0.005	0.112	n. d.	0.099	n. d.	0.031
Total flavonoids	0.068	0.530	0.197	0.850	0.019	0.728	0.007	0.423	0.009	0.302	n. d.	0.239	n. d.	0.085
Total polyphenols	1.17	2.22	2.86	3.37	1.65	2.16	0.849	2.08	0.980	2.37	0.870	1.58	0.808	1.35

The quantitative data (average of three analyses with S.D. in the range 2–3%) refer to plants grown in open air (Oa) and in the greenhouse (Gh). n.d., not detected.

samples, the flavonoids varied from 6 to 34% of the total polyphenol content, the last sampling being the lowest.

A greatest difference in flavonoid content was observed for samples collected at the end of May (9 and 12 days after transplanting). The flavonol content continuously decreased until the sample analysed 16 days after transplanting. This trend was observed, as well, for the last two samplings of June (20 and 27 days after transplanting); moreover, a presence–absence effect was evident in the greenhouse and open-air samples, respectively.

The trend of the two polyphenol classes identified and quantified by HPLC-DAD in lettuce leaves is shown in the histogram of Fig. 2. As shown, the highest content of both classes was in the sample collected 5 days after

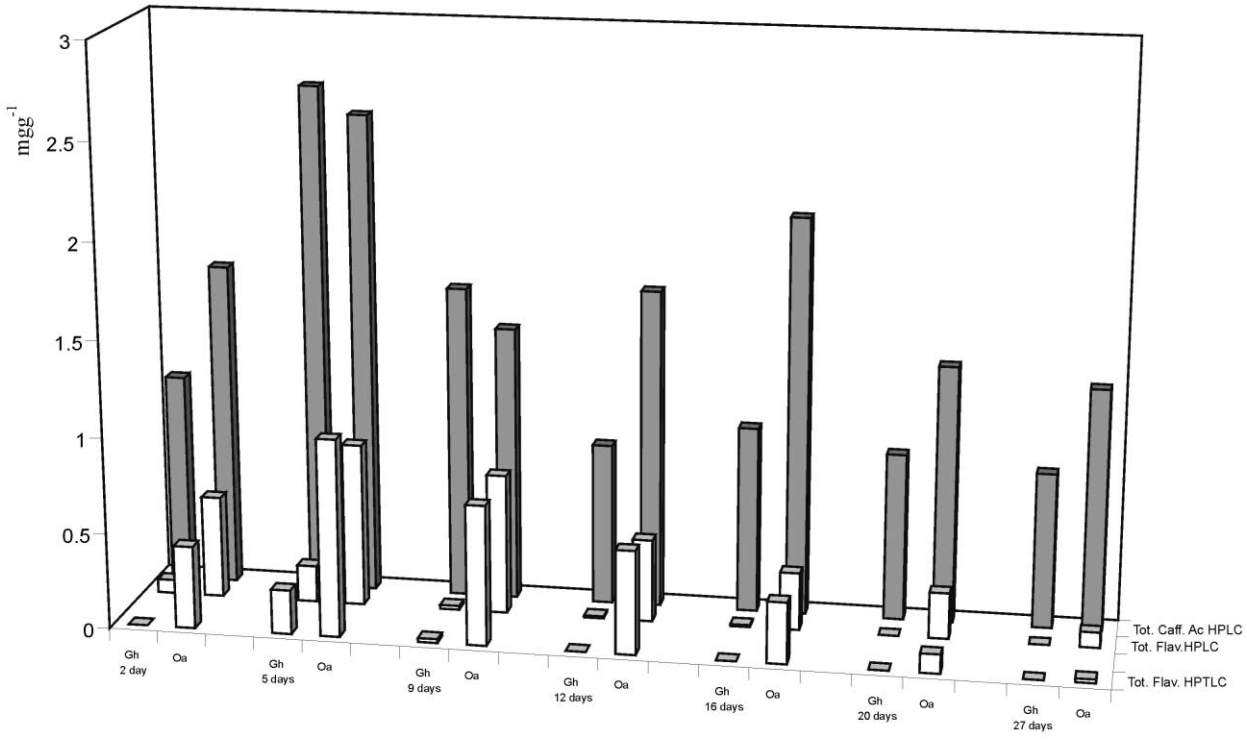

Fig. 2. Caffeic acid derivatives in lettuce leaves evaluated and characterised by HPLC/DAD and flavonoids quantified by HPLC/DAD and HPTLC, respectively. Data are expressed in mg g^{-1} of fresh weight. The quantitative data refer to plants growing in open air (Oa) and in the greenhouse (Gh).

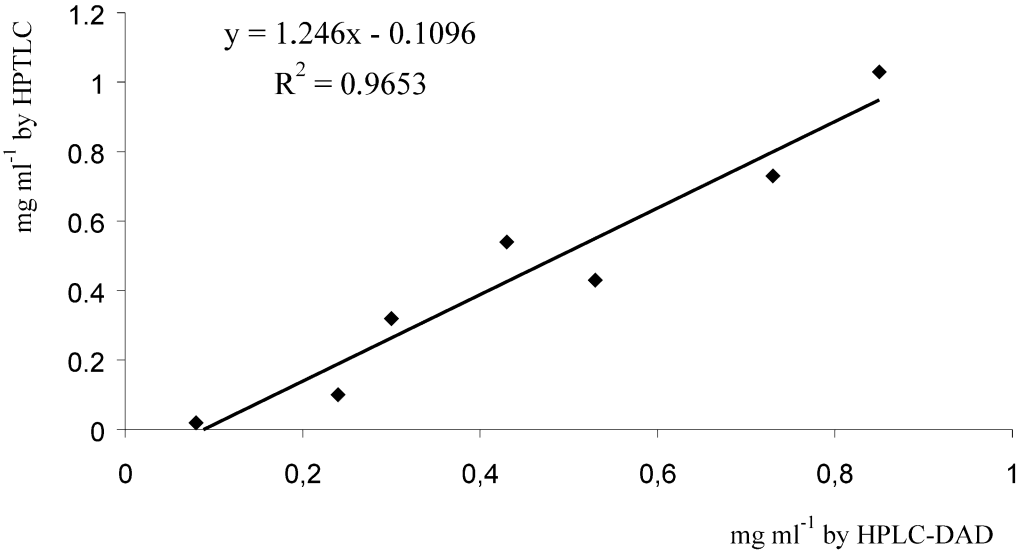

Fig. 3. Comparison between quantitative findings obtained by using HPTLC and HPLC techniques for total flavonol content of lettuce leaves in the open air samples.

transplanting: caffeic acid derivatives in greenhouse lettuce and flavonols in open-air samples. The ratios of flavonol content in open-air samples respect to the greenhouse ones range from about 4.5 (5 days after transplanting) to 43 (12 days after transplanting).

In order to optimise a rapid and sensitive method for flavonol screening in lettuce leaves, all the samples were analysed by HPTLC/densitometry. The quantitative results for flavonols, calculated as a sum of three spots and expressed as isoquercitrin, are also shown in Fig. 2. The HPTLC method is cost-effective because of the short analysis time and the low solvent consumption. The reproducibility of this technique allows a comparison with the data obtained by HPLC for the flavonol content. Although HPTLC is a sensitive method for lettuce flavonols, the phenolic acid results do not agree with those obtained by HPLC (data not reported) because, under these experimental conditions, 5-caffeoylquinic acid runs along with the solvent front and its quantitative determination is therefore affected by a systematic error.

Since the trend observed for flavonols is similar, a linear regression between these two techniques was performed. Fig. 3 shows the good correlation obtained and the high correlation coefficient (0.965). Finally an HPTLC method could be routinely used to analyse a high number of samples; in fact it requires a simple approach and a short analysis time.

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