Table 2
Induction times of lemon peel extracts measured by Rancimat

Solvent fraction	Induction time (h)
Petroleum ether fraction	3.6
Chloroform fraction	3.1
Ethyl acetate fraction	4.8
Butanol fraction	3.7
BHT	7.8
Control	4.1

HPLC has proved very useful in the analysis of lemon peel extract. In particular, the use of two detectors (diode-array detector and mass spectrometer) allowed us to obtain the UV–Vis spectrum and the mass spectrum of each compound. Thermospray mass spectrometry is suitable for the analysis of flavonoids because of the observation of ions that correspond to the quasi-molecular or molecular ion of the compound and of the aglycon. Therefore, it was possible to identify several glycosides present in the lemon peel together with other compounds of different structure. Lemon peel extracts were found to be characterized by the presence of several compounds belonging to four different groups of flavonoids: flavones-O-glycosides, flavones-C-glycosides, flavonols and flavanones, according to published data. Furthermore, several coumarins, a phenyl propanoid glucoside and some limonoids were found. These last compounds are of particular interest because they are related to the bitterness of the extracts and juices. A test on the antioxidant activities of the extracts was performed using a Rancimat apparatus. Comparison of the induction times of the different samples showed that only the EtOAc extract possessed antioxidant activities while the others did not.

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High-performance liquid chromatographic profiles of aloe constituents and determination of aloin in beverages, with reference to the EEC regulation for flavouring substances

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Abstract

Characteristic HPLC profiles of fresh and aged aloe solutions, detected at 360 and 220 nm, are presented and compared. Several aloe constituents (aloesin, aloeresin A, hydroxyaloin, aloin A and B and aloinoside A and B) were simultaneously separated and identified. The determination of aloin is described (detection limit 0.15 ppm) and discussed. In aloe-based alcoholic beverages, the aloins could not be detected, owing to their instability and degradation in solution; this is discussed in relation to the EEC Council Directive 88/388, which fixed the values of maximum allowable concentrations for aloin in food and beverages. Instead of aloin, other aloe compounds (e.g., aloeresin A or aloesin) should perhaps be used as an index of the presence of aloe in alcoholic beverages.

1. Introduction

The term aloe refers to the dried latex extracted from the leaves of several species of Liliaceae, plants which grow mainly in South and East Africa (Cape aloes from *Aloe ferox* or *A. arborescens* Mill., Socotrine aloes from *A. perryi* Bak.) and in the Caribbean region (Barbados or Curacao aloes from *A. vera* Linn.).

Aloe is widely used for manufacturing food products and beverages, pharmaceuticals and cosmetics because of its aromatic properties, bitter taste, the cathartic activity of anthraquinones and other pharmacological activities (such as emolliency, reduction of inflammation and acceleration of wound healing; it is not yet well understood which activity may be related to which component) [1].

Aloin, a mixture of two diastereoisomers, aloin A (configuration at C_{10}, $C_{1'}$: *S,S*) and aloin B (*R,S*) [2], is an anthrone C-glucoside component of aloes (it is also present in extracts from cascara bark, *Rhamnus purshiana* DC, and from other vegetable sources); the other main aloe components are aloesin (formerly named aloeresin B) [3] and aloeresin A [4]. Several other constituents of commercial aloes have also been described: aloe-emodin [5], homonataloin and nataloe-emodin [6], aloinoside A and B [7],

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aloenin A and B [8], 4-hydroxyaloin [9] and 5-hydroxyaloin [10].

Table 1 summarizes the names and absorbance spectral data as obtained from the cited references, and Fig. 1 depicts some of the corresponding structural formulae.

Aloin was included in the list of twelve restricted compounds for which, owing to their pharmacological activity, the EEC Council Directive 88/388 fixed the maximum allowable concentrations (MAC) in food products [11,12]. In Italy, the EEC Directive was enforced by a law [13] which established the MAC values but did not (as yet) publish the official method of analysis.

As far as analytical methods for aloin are concerned, the AOAC method [14] is not applicable to aloes; the pharmacopoeial methods [15,16] for drug preparations are based on TLC and are not suitable for food analysis. In fact, the Council of Europe [17], in 1981, reported the lack of methods for determining aloin in foods. Meanwhile, two papers have described HPLC methods for the analysis of food products and beverages for aloin: Yamamoto et al. [18] found aloin in Japanese candies with concentrations exceeding 1000 times the EEC MAC, whereas Zonta et al. [19] did not find aloin in any of the samples of bitter alcoholic beverages analysed.

Whereas TLC has often been used for aloe analysis [3,7,15,16], only a few papers have presented HPLC results for aloe constituents. Rauwald and Beil [20] recently reported the HPLC separation [with methanol–water (1:1) as isocratic mobile phase] of five anthraquinone derivatives from aloes: aloins (A and B), aloinosides (A and B) and 5-hydroxyaloin A, detected at 360 nm.

This paper describes and compares the HPLC profiles, detected at 220 and 360 nm, of fresh

Table 1
Some components of aloe, with names, absorbance spectral data (with corresponding ε or log ε values) and references

Name	Absorbance maxima (nm)[a]	Solvent	Ref.
Aloin (A and B)	208 (4.41), 255sh (3.77), 270 (3.91), 297 (3.96), 363 (4.05),		[6]
(formerly barbaloin)	261 (3.85), 269 (3.92), 297 (3.98), 362 (4.08)	MeOH	[8]
	269, 302, 379	KOH–MeOH	[8]
	260 (3.80), 269 (3.95), 298 (4.00), 360 (4.03)	MeOH	[21]
Aloesin	216 (4.31), 248 (4.21), 254 (4.23), 297 (3.96),	EtOH	[3]
(formerly aloeresin b)	212 (4.27), 244 (4.14), 252 (4.18), 296 (3.90)		[3]
Aloeresin A	228 (34 250), 243sh (25 900), 252 (25 000), 300 (37 960)	MeOH	[4]
Aloinoside (A and B)	269 (3.865), 296 (3.941), 360 (4.002)	MeOH	[7]
Aloenin B	205 (4.75), 224sh (4.48), 298 (4.44), 308 (4.45)	MeOH	[8]
5-Hydroxyaloin	Absorbance data not available		[10,20]
4-Hydroxyaloin	The absorbance spectrum is reported; the values of the absorbance maxima are not given and are difficult to be read from the figure. This substance was reported to be the main degradation product of aloin		[9]
Aloe-emodin	492, 510 (approximate values as deduced from the reported spectrum shape; aloemodin is the main product derived from aloin oxidation)	At pH 11	[5]
Homonataloin	222 (4.38), 250sh (3.85), 273sh (3.85), 294 (4.12), 347 (3.85)		[6]
Nataloe-emodin	232 (4.3), 260 (4.3), 290sh (4.0), 434 (3.89)		[6]

[a] Values in parentheses are ε or log ε.

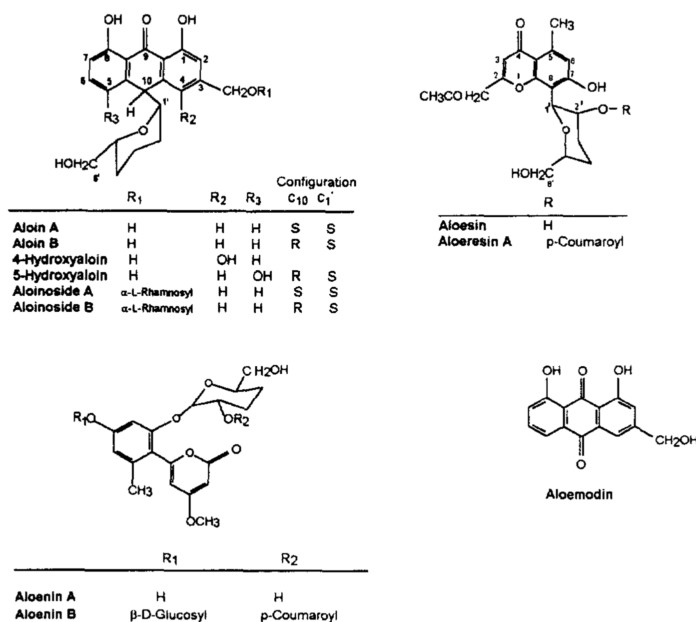

Fig. 1. Structural formulae of some aloe components.

and aged aloe solutions. In addition to the diastereoisomeric aloins A and B, many other peaks were simultaneously separated and the main components identified. The very short lifetime of aloin in aqueous–alcoholic solutions explains why aloin was not previously detected in beverages. As will be pointed out in the Discussion section, although the results presented here are satisfactory from the analytical point of view, they nonetheless lead to questions on the regulatory aspects.

2. Experimental

2.1. Apparatus

The chromatographic apparatus consisted of a pump module (Series 3 liquid chromatograph), a column oven (LC 100) and a variable-wavelength spectrophotometric detector (LC 55B) connected to a digital scanner (LC 55S) and a pen recorder (Model 56) (all from Perkin-Elmer, Norwalk, CT, USA). All chromatographic data were recorded and processed by means of Chromstar software (Bruker, Bremen, Germany).

The analytical column was a Supelcosil LC18 (250 × 4.6 mm I.D.) filled with a 5-μm body-porous stationary phase (Supelco, Bellefonte, PA, USA). The elution programme was isocratic [acetonitrile–water (18:82, v/v)] for 12 min, followed by a linear gradient to 35% acetonitrile in 20 min and isocratic again for 8 min; the run was then continued with a second linear gradient from 35% to 60% acetonitrile in 10 min. This was followed by a purge (95% acetonitrile for 10 min) and a re-equilibration step (to 18% acetonitrile) for 15 min before the next injection. The column was thermostated at 45°C, and the corresponding initial pressure drop was 9.2 MPa with a flow-rate of 1 ml/min. The spectrophotometric detector was set as required at either 360 or 220 nm.

2.2. Materials and methods

All solvents were of HPLC grade (Merck, Darmstadt, Germany). A crystalline sample of aloe (without specification of origin or purity) was a gift kindly supplied by Janousek (Trieste, Italy).

The powdered aloe (1 g) was mixed with water (100 ml) [15,16] or with aqueous–alcoholic mix-

tures (30%, v/v) or with absolute ethanol; the suspensions obtained were then centrifuged and diluted as required (e.g., 1:4). The solutions were kept in the dark at room temperature (about 20°C) or at 4°C for several weeks, to test their stability and changes; before the injection (injection volume 2 μl) they were filtered using HV 0.45-μm filters (Millipore).

Commercial samples of aloin of purity ca. 70% by TLC (Sigma, St. Louis, MO, USA) and >50% by TLC (Fluka, Buchs, Switzerland) were also used.

Commercial samples of alcoholic beverages (bitter and fernet) were purchased at random in grocery stores. They were filtered and injected directly for analysis (injection volume 3 μl).

3. Results and discussion

3.1. Qualitative analysis

This paper describes a reversed-phase gradient elution system (based on a water–acetonitrile mixture) which allows the simultaneous separation of many components of aloe. Characteristic HPLC profiles, detected at 220 nm, of fresh and aged aqueous solutions of aloe are shown in Fig. 2. Table 2 lists peak numbers, the corresponding spectral absorbance maxima and, where possible, their identification or tentative assignment, since some suggestions require further confirmation that could be obtained by preparative HPLC (which is planned as a continuation of this research).

Fig. 3 shows the shapes of the absorbance spectra of the eluting compounds, as recorded by means of the stop-flow method.

Aloins (peaks 14 and 17) were identified by using the available commercial standard (although impure and containing several other peaks characteristic of aloe chromatograms, which reveals it to be a natural, non-synthetic product).

The other main aloe constituents were known (from previous analytical results [3,4]) to be aloesin and aloeresin A, and these were found to correspond to the other two main peaks (3 and 11, respectively) on the chromatogram. Their elution order, also compared with aloins, was

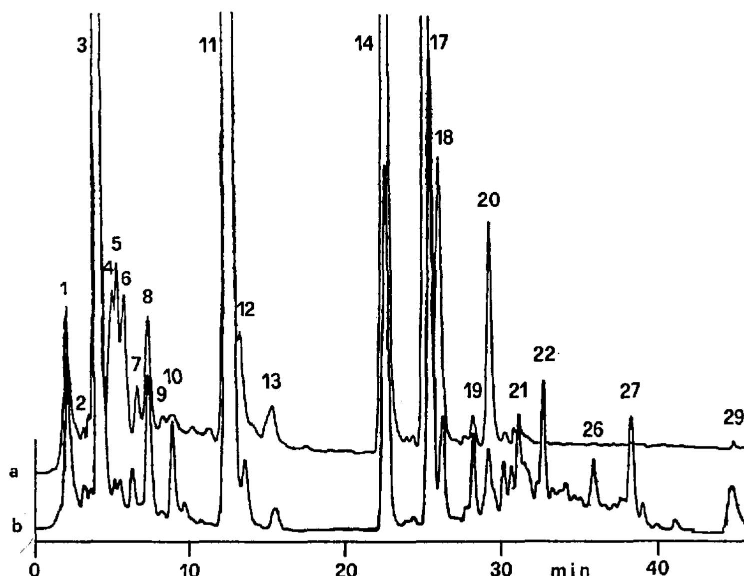

Fig. 2. HPLC profiles of aloe: (a) freshly prepared aqueous solution (superimposed above); (b) the same sample injected after 3 weeks (superimposed below). Detection wavelength, 220 nm; injection volume, 2 μl; recorder setting to 0.05 A.U.F.S. Other chromatographic conditions are described under Experimental. For peak identification, see Table 2.

Table 2
List of the main peaks of the chromatograms from aloe

Peak No.	Absorbance maxima (nm)										Peak identification
1	213,			253sh,			300,				
2	214,		245,	253,			298,				Aloesin
5	212,	228,		253,			304,				
6			246sh,	254,			303,				
10	211,		245sh,	254,			300				
11	212,	228sh,	243sh,	252,			303,				Aloeresin A
13	210,			253sh,	260sh,	269,	298,		359,		Hydroxyaloin (?)
14	210,			254sh,	262sh,	270,	300,		360,		Aloin B
17	210,			254,	262sh,	270,	299,		359		Aloin A
18				253,	261sh,	268,	298,		359,		Aloinoside B (?)
19				255,	265,	274,	290,	308,		365,	
20				255,	262sh,	269,	298,		359,		Aloinoside A (?)
21					265,	274,		308,		365,	
22	212,			255,		275,		305,		365,	
27	214,	228,			266,	275sh,	290sh,	306,		365,	

The absorption spectra of the peaks were recorded in the eluent solvent system (i.e., CH_3CN-H_2O) in the range 210–380 nm. Some data at the extremities of the explored wavelength range (210–220 and 370–380 nm) may be missing or uncertain owing to instrumental limitations.

consistent with that obtained by TLC [3]. Further, the absorbance spectral data corresponded with those in the literature. Although the spectral shapes of aloesin and aloeresin are remarkably similar, they show very different molar absorptivities, since the bands near 250 nm of

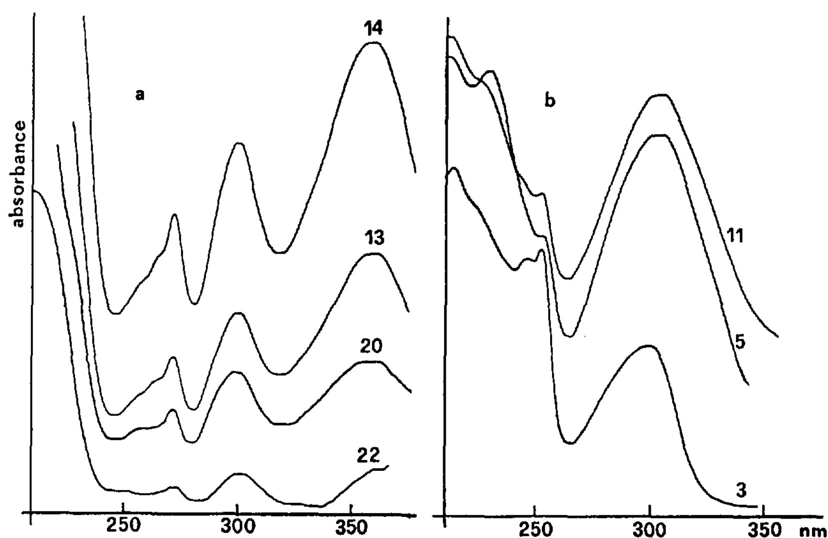

Fig. 3. Absorption spectra of some of the eluted peaks as recorded by means of the stop-flow method. Absorbances of the different spectra are not to scale. Spectral identification numbers refer to the peak numbers on the chromatograms. (a) "Aloin type" spectra with three major bands, around 270, 300 and 360 nm; (b) other spectra, lacking the band around 360 nm, with an absorbance and (benzenoid) around 300 nm. Detailed values of absorbance maxima are given in Table 2.

aloesin are higher than that near 300 nm; the opposite applies to aloeresin (compare the absorbance spectral data in Tables 1 and 2 and the spectral shapes in Fig. 3b).

Peak 18 (which is incompletely resolved and not pure) has an absorption spectrum similar to but not identical with that of peak 20. These two peaks should be the aloinoside A and B, respectively (and their heights decrease with time). Their tentative assignment is mostly based on the fact that when we reproduced a solvent system similar to that used by Rauwald and Beil [20] [CH_3OH–H_2O (1:1), but with a flow-rate reduced to 0.7 ml/min] and used the same detection wavelength (360 nm), a chromatogram of aloe very similar to theirs was obtained (and the pair of peaks 17 and 18 was much better separated). Further, the absorbance maxima of peak 20 correspond to those reported for the aloinoside [7].

The chromatogram of the aged (3 weeks) aloe solution is reported superimposed (profile b) in Fig. 2. Several changes were evident on comparing the two chromatograms: there was a large decrease, due to degradation, of the aloins (A and B, peaks 17 and 14, respectively) and of the supposed aloinosides (peaks 18 and 20); a corresponding increase in peaks 10, 21, 22, 26, 27 and 29, which should correspond to degradation products, was observed. Graf and Alexa [9] reported 4-hydroxyaloin (eluting before aloin) to be the main decomposition product of aloin, but this could not be clearly confirmed here. Rauwald and Beil [20] reported 5-hydroxyaloin (a component to which taxonomic significance was attributed) as another peak eluting before aloins and characteristic of aloe samples. The spectral shape and the elution position of peak 13 in the chromatogram suggest that it could perhaps be a hydroxyaloin, but this is a highly uncertain speculation.

At 360 nm, since many components of the mixture absorb very weakly, the chromatogram is simplified and fewer peaks are detectable; in fact, this is a selective wavelength for aloins (and other anthraquinone derivatives), but important information about other components is missing, as shown in Fig. 4,where the heights of the peaks attributable to aloesin and aloeresin are greatly reduced. Fig. 4 also shows, superimposed for comparison, the chromatograms obtained at 360 nm by using a fresh and an aged (3 weeks) aloe solution (profiles a and b, respectively).

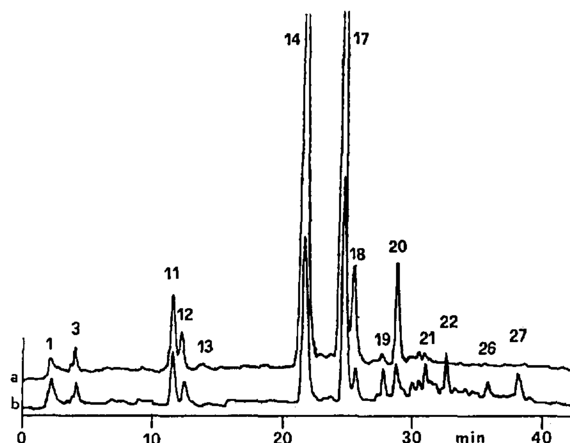

Fig. 4. Superimposed chromatograms of fresh (profile a, above) and aged (profile b, below) aloe solutions as detected at 360 nm. Chromatographic conditions and peak numbers as in Fig. 2.

3.2. *Quantitative analysis*

The HPLC determination of aloin has been described previously [19]. At 360 nm, which corresponds to an absorbance maximum of aloin, the log ε value (4.03) reported in the literature [21] was used for obtaining the concentration of a standard solution of pure aloin (obtained by preparative HPLC). The linearity of the calibration graph was verified within the range 0.15–60 ppm. The detection limit was 0.15 ppm for each of the two aloin diastereoisomers (with a signal-to-noise ratio of 3) [19].

The commercial standard (Sigma) was then analysed and the aloin concentration was found to be $73.1 \pm 0.7\%$ ($n = 7$), a value close to that declared on the label by the manufacturer (ca. 70% by TLC). The commercial unavailability (as far as we know) of highly pure aloin standards may lead to controversial results when official analytical controls of aloin concentration in food and beverages are required.