Quantitative HPLC Analysis of Acids in Brazilian Cachaças and Various Spirits Using Fluorescence Detection of Their 9-Anthrylmethyl Esters

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An analytical procedure for the separation and quantification of 17 short-chain, medium-chain, and long-chain acids in cachaças and various spirits has been developed involving C18 solid phase extraction, derivatization with 9-anthryldiazomethane, and reverse phase HPLC using fluorescence detection. The limit of detection was between 5 and 15 fmol, whereas the recovery of nonanoic acid as internal standard was >95%. Relative standard deviation values for reproducibility were between 0.09 and 20.4%, and repeatability was between 0.05 and 11.3%.

Keywords: *HPLC; acids; fluorescence; 9-anthrylmethyl esters; spirits*

INTRODUCTION

Gas chromatographic determination of acids in spirits as their corresponding esters has been frequently reported (Dugar, 1995; Gallart et al., 1997; Galli and Antonelli, 1995; Guymon, 1963; Hageman and Roerade, 1990; Liebich et al., 1970; Masuda et al., 1985; McCallery, 1989; Morales, 1996; Nascimento et al., 1998a,b; Metcalfe and Schmitz, 1961; Nykanen et al., 1968; Quin and Hobbs, 1958; Schlenk and Gellerman, 1960; Stevens and Martin, 1965; Stoffel et al., 1959). Usually, liquid−liquid extraction precedes esterification to methyl or ethyl esters. The improved performance of capillary columns coated with polar phases coupled to direct injection allows detection and quantification of aliphatic and some aromatic acids in spirits at levels below milligrams per liter (Cantagrel, 1992). A much more sensitive analysis of acids at the femtomole level was developed by Barker et al. (1980) and was based on fluorescence detection of their 9-anthrylmethyl esters. This method proved to be particularly suitable to assay acids in biological matrices, and even picomoles could readily be detected (Batty and Pazouki, 1987; Ichinose et al., 1984; Imaoka et al., 1983; Nimura and Kinoshita, 1980; Shimomura et al., 1986; Yasaka and Tanaka, 1994). In view of our interest in the analysis of traces of acids in alcoholic beverages, we have applied this fluorescence technique for the first time to cachaças and various spirits and found that the method was more selective and accurate with respect to traditional gas chromatographic techniques with flame ionization detection.

EXPERIMENTAL PROCEDURES

Apparatus. HPLC separations were carried out on a Shimadzu HPLC model LC-10AD equipped with a Rheodyne injector model 7125, a C-18 Supelcosil column (25 × 4.6 mm; 5 μm), and a Shimadzu RF-530 fluorescence detector with a flow cell volume of 12.0 μL. The data were evaluated using a Shimadzu C-R1A data processor.

Table 1. Calibration Curves ($y = a + bX$) for the Analysis of Acids as Their 9-Anthrylmethyl Esters

acid	*a*	*b*	*r*	SD
acetic (C1)	0.0098	1.11	0.998	0.013
propionic (C3)	0.123	71.29	0.998	0.133
isobutyric (IC4)	0.051	53.46	0.996	0.063
butyric (C4)	0.007	27.7	1.00	0.009
csovaleric (IC5)	0.016	12.71	0.998	0.024
valeric (C5)	0.012	12.82	0.997	0.031
benzoic (C6:3)	0.018	0.463	0.994	0.029
caproic (C6)	0.009	40.3	0.996	0.026
heptanoic (C7)	0.009	0.098	0.999	0.012
caprylic (C8)	0.017	0.154	0.996	0.026
capric (C10)	0.012	0.319	0.999	0.030
lauric (C12)	0.037	0.158	0.994	0.044
myristic (C14)	0.036	0.125	0.992	0.041
palmitic (C16)	0.034	0.178	0.996	0.039
linoleic (C18:2)	0.018	0.101	0.997	0.022

Reagents. Pure fatty acids, used as standards, were purchased from Aldrich (Milwaukee, WI); ethanol, acetone, and dichloromethane (analytical grade) from Merck (Rio de Janeiro, Brazil); and methanol and acetonitrile (HPLC grade) from Mallinckrodt (Xalostoc, Mexico). Water was purified by a Milli-Q system (Millipore, Bedford, MA). 9-Anthryldiazomethane (9-ADAM) was prepared according to the literature method (Nakanya et al., 1967).

Preparation of Standard Solutions of 9-Anthrylmethyl Esters of Fatty Acids. A stock solution of 1.00 mM mL^{-1} of each constituent of a mixture of acids (acetic, propionic, isobutyric, butyric, isovaleric, valeric, benzoic, isocaproic, caproic, heptanoic, caprylic, capric, lauric, myristic, linoleic, palmitic, heptadecanoic, and stearic) was prepared in acetone (Imaoka et al., 1983). Solutions of 9-anthrylmethyl esters, at appropriate concentrations (from 0.10 to 100 μM mL^{-1}) to determine calibration curves, were prepared by addition of 2.00 mL of a 9-ADAM solution (0.1% w/v in acetonitrile, stored at −10 °C) to an adequate volume of the stock solution of the fatty acids mixture and were incubated at room temperature for 35 min in the dark. An aliquot (20.0 μL) was injected for each HPLC run. Data for calibration curves are collected in Table 1.

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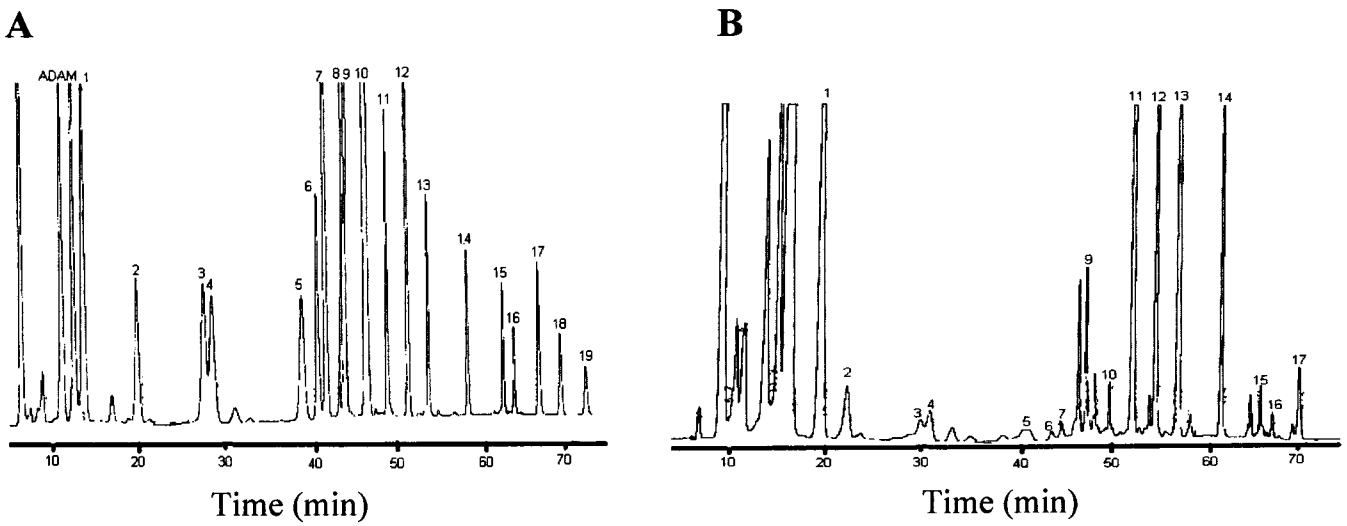

Figure 1. (A) HPLC analysis of a standard mixture of acids as their 9-anthrylmethyl esters. (B) HPLC analysis of acids in cachaça sample 12 as their 9-anthrylmethyl esters. Chromatographic conditions: see Experimental Procedures. Peak identification: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = butyric acid; 5 = isovaleric acid; 6 = valeric acid; 7 = benzoic acid; 8 = isocaproic acid; 9 = caproic acid; 10 = heptanoic acid; 11 = caprylic acid; 12 = nonanoic acid (IS); 13 = capric acid; 14 = lauric acid; 15 = myristic acid; 16 = linoleic acid; 17 = palmitic acid; 18 = heptadecanoic acid; 19 = stearic plus oleic acids.

Samples. To study a representative number of cachaças (Nascimento et al., 1998a), commercially available samples for export and for local consumption (regular samples) were collected from different regions of Brazil. The samples were selected according to preferences referring to both traditional production and consumers' acceptance. A minimum of three samples of each brand was assayed. Cachaças are labeled throughout as type a, export (7 samples), and type b, regular (10 samples). There is no relation between chemical sensorial qualities of cachaças. Type a: Caranguejo (CE), 51 (SP), Jamel (SP), Ypioca Ouro (CE), Velho Barreiro (SP), Nêga Fulô (RJ), Mulher Rendeira (RJ). Type b: Cavalinho (SP), Germana (MG), Kariri (CE), Massayo (AL), Marquesi (SP), Sapupara (CE), Baronesa (SC), Salinas (MG), Vila Velha (SP), Velha Aroeira (MG). States in Brazil: SP, São Paulo; SC, Santa Catarina; MG, Minas Gerais; CE, Ceará; RJ, Rio de Janeiro.

Various other spirits were investigated as well: cognac Courvoisier (France); grappa Euganea (Italy); whiskeys Johnny Walker Red Label (Scotland), Jack Daniel's (U.S.), Jim Beam (U.S.), Chivas (Scotland), Buchanan's (Scotland), Passport (Scotland); rum Havana Club (Cuba); pisco Control (Chile); and Brazilian whiskeys Black Jack and Mark One.

Sample Extraction. All samples were preconcentrated by solid phase extraction (SPE) using Supelclean ENVI-18 (Supelco, Bellefonte, PA). Each cartridge was washed with 2 mL of methanol and 2 mL of water/ethanol (60:40 v/v) at a pH of 4.5. Samples (50.0 mL), containing 25.0 μg mL^{-1} nonanoic acid as internal standard, were eluted with 2 mL of dichloromethane at a flow rate of 5 mL min^{-1}.

Fluorescent Labeling of Samples. A fraction of the eluate (200 μL) was treated with 2.0 mL of a solution of 9-ADAM (0.1% w/v in acetonitrile). The same procedure, as described above for the preparation of standard solutions, was applied.

Conditions for HPLC. Methanol and water were used as eluents, and gradient conditions were as follows: methanol/water 75:25 (v/v) isocratic during 35 min (1.0 mL min^{-1}), from 75:25 to 90:10 during 5 min, 90:10 isocratic during 20 min (1.2 mL min^{-1}), from 90:10 to 100:0 during 5 min, 100:0 isocratic during 5 min (1.2 mL min^{-1}), from 100:0 to 75:25 during 5 min (1.0 mL min^{-1}).

RESULTS AND DISCUSSION

Quantitative esterification of the acids is reportedly achieved in the presence of a 12-fold excess of ADAM (Shimomura et al., 1986). Although excess reagent is usually decomposed using acetic acid, we did not follow this procedure because we wished to assay acetic acid in the samples as well. Thus, fluorescent decomposition products were evident in the early part of the HPLC chromatograms. However, no interference with the analysis of the 9-anthrylmethyl esters of the acids was noted. Quantitation was achieved using nonanoic acid as internal standard, and fluorescence was monitored at 440 nm with excitation at 360 nm (room temperature). Figure 1 displays an HPLC chromatogram of the mixture of standards and an HPLC analysis of a representative cachaça sample. Acids such as benzoic, linoleic, and stearic, which can be detected with only low sensitivity in spirits by GC-FID (Nascimento et al., 1998a), could be efficiently quantified via fluorescence detection. As can be observed, various 9-anthrylmethyl esters are nicely separated. Isovaleric and valeric acids could be distinguished only using gradient elution, whereas isobutyric/butyric and linoleic/oleic acid still coelute. Hence, in Tables 3 and 4, the sum of the concentrations of stearic and oleic acid is given. The detection limits reach the femtomole level, which significantly exceeds that of gas chromatographic methods

Table 2. Reproducibility and Repeatability of the Analysis of Acids in Spirits as Their 9-Anthrylmethyl Esters[a]

	reproducibility		repeatability	
acid	mean[b] ± SD	RSD (%)	mean[c] ± SD	RSD (%)
acetic (C1)	112.1 ± 0.100	0.090	90.87 ± 0.044	0.048
propionic (C3)	0.118 ± 0.002	1.695	0.091 ± 0.004	4.396
isobutyric (IC4)	0.251 ± 0.021	8.366	0.208 ± 0.009	4.327
butyric (C4)	0.147 ± 0.030	20.40	0.097 ± 0.011	11.34
isovaleric (IC5)	0.195 ± 0.001	0.513	0.182 ± 0.008	4.396
valeric (C5)	0.033 ± 0.002	6.060	0.030 ± 0.001	3.333
benzoic (C6:3)	0.030 ± 0.002	6.666	0.020 ± 0.001	5.000
caproic (C6)	0.524 ± 0.009	1.717	0.443 ± 0.003	0.677
heptanoic (C7)	0.110 ± 0.004	3.636	0.097 ± 0.004	4.124
caprylic (C8)	2.022 ± 0.075	3.709	1.980 ± 0.042	2.121
capric (C10)	2.010 ± 0.017	0.846	1.930 ± 0.051	2.642
lauric (C12)	0.676 ± 0.032	4.734	0.623 ± 0.009	1.445
myristic (C14)	0.111 ± 0.003	2.703	0.137 ± 0.002	1.460
palmitic (C16)	0.241 ± 0.003	1.245	0.218 ± 0.012	5.505
linoleic (C18:2)	0.024 ± 0.001	4.167	0.031 ± 0.001	2.703

[a] Isocaproic, heptadecanoic, and stearic acids are not included, as they are not present in all samples studied. [b] $N = 5$, injected twice. [c] $N = 10$.

Table 3. Quantitative Analysis of 9-Anthrylmethyl Esters of Acids in Cachaças Types a and b[a]

sample	C2	C3	IC4	C4	IC5	C5	C6:3	C6	C7	C8	C10	C12	C14	C16	C17	C18:2	A[b]
Cachaças Type a																	
01	27.4	0.100	0.282	0.253	0.511	0.089	<DL[c]	0.803	0.133	2.97	3.20	2.87	0.84	1.18	<DL	0.044	0.925
02	57.0	0.166	0.221	0.368	0.850	0.148	0.189	0.728	0.122	1.57	1.78	1.139	0.505	0.377	<DL	<DL	0.267
03	50.5	0.196	0.235	0.432	0.693	0119	0.010	0.730	0.135	0.910	1.24	0.985	0.572	0.538	0.389	0.389	0.052
04	60.2	0.159	0.235	0.352	0.791	0.123	0.018	0.857	0.013	1.31	1.43	1.06	0.916	0.533	<DL	0.237	0.054
05	31.4	0.196	0.153	0.357	0.645	0.118	0.012	0.808	0.101	0.995	1.11	0.739	0.130	0.637	<DL	0.127	0.058
06	53.8	0.235	0.140	0.350	0.740	<DL	0.052	0.512	0.126	2.13	2.43	1.77	0.455	0.789	0.039	0.178	0.063
07	88.9	0.389	0.174	0.432	0.586	0.134	0.023	0.586	0.143	1.42	1.57	0.878	0.217	0.274	<DL	0.009	0.569
X	52.7	0.206	0.206	0.363	0.688	17.1	0.051	0.718	0.110	1.62	1.82	1.35	0.519	0.618	0.214	0.164	0.284
Cachaças Type b																	
01	95.0	0.380	0.388	0.380	0.493	<DL	0.062	0.347	0.056	1.11	3.03	2.59	1.40	2.03	<DL	0.279	0.630
02	112	0.27	0.270	0.358	0.730	<DL	<DL	0.188	0.067	0.780	1.44	1.29	1.59	2.59	<DL	0.289	0.921
03	168	0.133	0.132	0.530	0.730	0.267	<DL	0.373	0.079	0.730	1.68	1.29	0.700	2.49	0.003	0.344	0.570
04	92.9	0.207	0.161	0.321	0.966	0.267	0.061	0.864	0.001	1.06	1.48	1.42	1.15	1.95	<DL	0.435	0.352
05	367	0.337	0.374	0.295	0.831	0.089	0.213	0.861	0.238	1.67	1.97	1.38	1.21	0.832	<DL	0.050	0.076
06	72.6	0.111	0.165	0.267	0.351	0.068	0.001	0.605	0.117	1.06	1.33	1.21	0.668	2.39	<DL	0.827	<DL
07	69.8	0.148	0.335	0.452	0.792	0.176	0.013	0.605	0.058	0.623	0.760	0.374	0.310	0.392	<DL	0.084	0.015
08	40.0	0.130	0.387	0.291	0.852	0.122	0.011	0.676	0.145	1.36	0.990	1.93	0.151	0.534	<DL	0.226	0.040
09	56.6	0.304	0.467	0.374	0.772	0.170	0.085	0.875	0.223	1.25	1.35	0.94	0.186	0.249	<DL	0.100	0.004
10	81.5	0.265	0.110	0.352	0.553	<DL	<DL	0.652	0.058	0.900	2.83	2.27	1.23	4.12	<DL	0.414	0.773
X	116	0.229	0.279	0.362	0.707	0.166	0.064	0.605	0.104	1.05	1.68	1.47	0.859	1.76	0.003	0.305	0.208

[a] Values in mg/100 mL of absolute ethanol. [b] Sum of stearic and oleic acids. [c] <DL, less than detection limit (5−15 fmol).

Table 4. Quantitative Analysis of 9-Anthrylmethyl Esters of Acids in Various Spirits[a]

sample	C2	C3	IC4	C4	IC5	C5	C6:3	C6	C7	C8	C10	C12	C14	C16	C17	C18:2	A[b]
01	45.7	0.090	0.243	0.069	0.966	<DL[c]	0.017	0.381	0.071	2.03	2.57	4.19	1.16	0.350	<DL	0.150	0.135
02	26.7	0.008	0.339	0.160	0.792	<DL	0.046	0.708	0.070	3.31	4.73	5.65	1.99	0.980	<DL	0.375	0.07
03	26.0	0.330	0.107	0.053	0.633	<DL	<DL	1.66	0.248	4.95	5.93	8.11	2.09	0.883	<DL	0.404	0.367
04	28.9	0.233	0.139	0.348	0.740	0.125	0.025	0.512	0.126	2.13	2.43	1.77	0.455	0.789	0.039	0.178	0.063
05	41.3	0.106	0.178	0.178	0.761	<DL	<DL	0.564	0.027	2.44	3.11	4.31	1.58	1.01	<DL0	0.017	0.169
06	35.0	0.078	0.313	0.104	0.653	<DL	0.011	0.63	0.114	1.38	1.97	2.85	1.10	0.38	0.003	<DL	0.332
07	33.4	0.254	0.291	0.145	0.632	<DL	0.069	0.801	0.253	3.85	4.40	6.77	2.65	1.76	0.001	0.325	0.080
08	45.7	0.056	0.312	0.156	0.827	<DL	0.010	0.704	0.258	3.03	3.47	5.10	1.94	1.77	<DL	0.310	0.233
09	24.6	0.184	0.575	0.258	0.905	0.102	0.038	1.40	0.372	2.39	2.37	1.88	0.286	0.693	<DL	0.018	0.084
10	37.4	0.252	0.363	0.211	1.10	0.100	0.044	1.80	0.496	1.47	0.821	0.493	0.176	0.567	<DL	0.062	0.150
X	34.5	0.159	0.286	0.168	0.801	0.109	0.032	0.916	0.203	2.69	3.18	4.11	1.34	0.918	0.010	0.204	0.168

[a] Values in mg/100 mL of absolute ethanol. [b] Sum of stearic and oleic acids. [c] <DL, less than detection limit (5−15 fmol).

(nanomole level). Moreover, characteristic luminescent properties of each compound allow for selectivity.

The calibration curves for 9-anthrylmethyl esters, recorded between 2.5 and 150 pmol, showed correlation coefficients close to unity (Table 1). The recovery of the method was estimated with the help of the internal standard (Nascimento et al., 1998a; Shimomura et al., 1986). Table 2 shows the results for the reproducibility [relative standard deviations (RSD) between 0.09% (acetic) and 20.4% (butyric)] and the repeatability [RSDs between 0.05% (acetic) and 11.3% (butyric)]. The precision of the method was evaluated by repetitive analyses of a single sample over 2 days.

The results of the quantitative analyses of cachaças type a, cachaças type b, and spirits are collected in Table 3. The qualitative profiles are similar for all beverages studied, but significant quantitative differences are noted. The total content of acids, which appears to be higher in cachaças with respect to other spirits, should, according to Brazilian legislation, be limited to 150 mg (as acetic acid)/100 mL of absolute ethanol (Ministério da Agrilcultura, 1974). Cachaças type a conform to the law, but two of the cachaças type b (Table 3, samples 03 and 05) exceed the legal limit. The total content of acids in cachaças of type b is lower than that determined for the cachaças of type a. The most abundant acid in all alcoholic beverages is acetic acid, up to 90−95% of the total content of acids found. Tables 3 and 4 show quantitative data of acids in Brazilian cane sugar spirits and other alcholic beverages. These values are in the same order of magnitude reported for acids in whiskeys, rums, and cognacs (Masuda et al., 1985; Nykanen et al., 1968; Stevens and Martin, 1965).

Identification of individual 9-anthrylmethyl was readily achieved by comparison of the retention times with those of standards and by standard addition.

Analysis of fatty acids as 9-anthrylmethyl esters in complex matrices appears to be successful (Baker et al., 1980; Batty and Pazouki, 1987; Ichinose et al., 1984; Imaoka et al., 1983; Shimomura et al., 1986), and our study proved for the first time the suitability for analysis of spirits. The method takes advantage of both the high selectivity and sensitivity of the fluorescent 9-anthrylmethyl esters (Baker et al., 1980; Ichinose et al., 1984). The detection limit was estimated to be in the low femtomole range: 5 fmol (benzoic acid) to 15 fmol (acetic acid) (signal-to-noise ratio of 3).

CONCLUSION

Quantitative analysis of acids based on fluorescence of 9-anthrylmethyl esters offers clear advantages of sensitivity and selectivity with respect to gas chromatographic analysis of esters using nonselective detection. Fluorescent labeling of acids using 9-anthryldiazomethane is readily achieved, whereas the 9-anthrylmethyl esters of 16 acids, including short-chain, medium-chain, and long-chain acids, could be completely separated and quantified. Preliminary results indicate that the procedure can be extended to quantitative analysis

of a variety of acids, including succinic, lactic, oxalic, malic, and formic acids, which usually escape determination by direct gas chromatographic analysis.

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