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Determination of riboflavin, flavin mononucleotide and flavin–adenine dinucleotide in wine and other beverages by high-performance liquid chromatography with fluorescence detection

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Abstract

A new direct HPLC method with fluorescence detection has been developed for the routine analysis of riboflavin, flavin mononucleotide and flavin–adenine dinucleotide, in wines and other beverages. These compounds are the main agents responsible for the ''taste of light'' that some white wines and other beverages develop when they are exposed to the light, due to the formation of sulfur compounds that produce an onion/garlic odor. A Hewlett-Packard 1100 gradient liquid chromatograph with 1046A fluorescence detector was used. To improve the selectivity, each compound was monitored to fit the best $\lambda_{excitation}/\lambda_{emission}$ (265/525 nm). A 500 nm cut-off filter was used. The column was a Hypersil C_{18} ODS, 200×2.1 mm, 5 μm particle size. The volume injected was 20 μl. A constant flow-rate of 0.6 ml/min was used with two solvents: solvent A, 0.05 *M* buffer NaH_2PO_4 at pH=3.0 with H_3PO_4 and solvent B, acetonitrile. The precision, linearity and sensitivity of this method have been established.

Keywords: Wine; Fruit juices; Beer; Food analysis; Vitamins; Flavin–adenine dinucleotide; Riboflavin; Flavin mononucleotide; Nucleotides

1. Introduction

The occurrence of an unpleasant taste, variously described as ''skunky'', ''cooked cabbage'' and ''onion-garlic'' following exposure to light has been reported in many beverages such as sparkling and white wines, beer, cider, milk and fruit juices. The detrimental effect of light on the aroma of these beverages is connected with different chemical processes for which riboflavin (RF) is required.

The appearance of an off-flavor described as ''sunlight flavor'' or ''reduced flavor'' in wines is linked to the photo-generation of thiols (methylmercaptan, H_2S) and dimethyldisulfide in wine exposed to light of wavelength below 450 nm [1–4]. This off-flavor is caused by photo-degradation of methionine and cysteine in the presence of RF, which acts as a photosensitizer and oxidating agent in wine [3,4], with a mechanism that is very similar to that reported for milk, where the main oxidation product is methional [5]. The ''sunlight flavor'' is reported to be produced easily in clear bottles of Chardonnay and Pinot gris wines with a RF content over 200 μg/l, when exposed to reflected light for two or three weeks, while a concentration below 100 μg/l is considered safe for such wines [6].

The photoactivation of riboflavin in beer leads, by many pathways, to the formation of carbonyl compounds giving rise to undesired ''oxidized-stale'' off-flavors or to the formation of mercaptans that are

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responsible for the ''sunstruck flavor'' or ''light-struck flavor'' [7,8]. The main compound produced is 3-methyl-2-butene-1-thiol (MBT), one of the most potent odor substances known, having a flavor threshold in beer of 7 ng/l [9]. MBT is formed exclusively by photolysis of iso-α-acids in the presence of RF and sulfur-containing amino acids [10–12]. RF is involved in the rate-determining step and therefore the intensity of the light-struck odour in beer bottled in clear or green glass exposed to visible light (400–500 nm) steadily increases with the content of RF, particularly at the low concentrations of RF that occur in beer [9,13].

Much research has been devoted to the control of the lightstruck flavor. In beer, this has been obtained through packaging technology, use of chemically modified hop bitter acids, use of antioxidants, removal of RF by actinic radiation during processing, etc. [12,14–17], while in wine control has been achieved by the use of cupric cation, dithionite anion and tannins [3,4]. Fining with bentonite has proven to be particularly effective in reducing RF levels in wine [6]. Display practices such as bottling in coloured glass containers and protecting from bright light do have a fundamental protective role [18], and it has been reported that RF concentration can be a good index of the likely degree of deterioration in the flavor of alcoholic beverages (white wines, sparkling wines and beers) on exposure of bottles to light [19]. Since marketing strategies require the sale of some beers and wines in clear (or green) glass bottles, it is important to develop methods to evaluate the susceptibility of a beverage to light. The concentration of RF appears to be a key factor in assessing the risk of the appearance of a light-struck flavor.

The principal forms of riboflavin (vitamin B_2) found in nature are flavin mononucleotide (FMN) and flavin–adenine dinucleotide (FAD). Free RF is also naturally present in raw and processed fruits [20,21] and fermented beverages. FAD and FMN can be converted to RF prior to quantitation, in order to obtain the total riboflavin content (TRF).

TRF was reported to be 50–70 μg/l in grape and in must, the content in wine rises to 110–250 μg/l during fermentation and it can be further enriched (160–318 μg/l) for wines left in contact with yeast for four to six days after fermentation is completed [22,23]. RF contents in beers have been reported typically in the range 100–575 μg/l [9,19,24–26]. Brewers' yeast produces RF as an extracellular by-product. RF is produced by growing cells only, with a production rate proportional to growth rate of the yeasts in the exponential phase, followed by a depletion in the stationary phase [26].

High-performance liquid chromatography (HPLC) with a reversed-phase column has been successfully applied to the analysis of vitamin B_2 in many different foodstuffs and biological matrices, since it provides sensitivity and selectivity and overcomes some of the problems associated with chemical methods [27]. Most methods have aimed to analyse RF or TRF together with other B vitamins [28–31], but it is possible to design a more specific method suitable for the simultaneous detection of RF, FAD and FMN [32–35]. Two specific HPLC procedures for RF in alcoholic beverages are described in the literature. Moll and Charalambous [19] developed a method for the reversed-phase HPLC–fluorescence determination of RF in wine, cider and beer by direct injection and Pichler [6] proposed an HPLC–UV method for the measurement of free RF in white wine. Both methods provide a quick measurement of free RF by direct injection, under the assumption – not verified up to now by direct measurements – that FMN and FAD were insignificant in these beverages.

Pichler [6] proposed a simple standardized method for inducing the sunlight flavor through exposure of 100 ml of wine to direct light. This method can identify wines that are likely to develop the defect when not stored under proper conditions, and it can be applied to beers and other beverages. In such conditions fruit juices fortified with vitamin B_2 at the level of a few mg/l can also easily develop a sunlight flavor. By adding a known amount (1 mg/l) of RF, or FAD or FMN to a wine that did not produce this flavor, we verified that each form of vitamin B_2 was capable of producing an off-flavor aroma, as can be expected considering the similarity between their molecular structures. This suggested the necessity for the development of a suitable method for the simultaneous evaluation of all B_2 vitamins.

A new HPLC–fluorescence method with direct injection of the sample has been developed for the specific analysis of riboflavin, FMN and FAD in wines, beer and fruit juices.

2. Experimental

2.1. Standards

FMN and FAD (both of 97% purity) were purchased from Sigma (St. Louis, MO, USA) and RF (98% purity) was from Aldrich (Milwaukee, WI, USA). Standard solutions were freshly prepared daily in dimmed light, amber glass bottles were used and the solutions were stored in a refrigerator (4°C) because B_2 vitamins are light-sensitive. Riboflavin solution was diluted with 20% of acetonitrile and 80% of the solvent A (0.05 *M* buffer NaH_2PO_4 at pH=3.0 with H_3PO_4). FMN and FAD were diluted with doubly distilled water. All the standards were passed through Millex-GV^{13}, 0.22 μm disposable filters (Millipore) before being injected into the column.

2.2. Equipment

A Hewlett-Packard (HP) 1100 gradient liquid chromatograph with an HP-1046A fluorescence detector was used (Hewlett-Packard, Waldbronn, Germany) and a syringe loading injection valve with a 20-μl loop model 7010 (Rheodyne, Cotati, CA, USA). The best selectivity for each compound was reached at 265/525 nm ($\lambda_{excitation}/\lambda_{emission}$) using a 500 nm cut-off filter. The slit width combination to reach the best *S*/*N* ratio was obtained with a 2×2 mm slit on the excitation side and two 4×4 mm slits on the emission side. A higher sensitivity was required for FAD and FMN, therefore the gain of the photomultiplier was set to 17 from the injection up to 6.5 min, then it was reduced to 13 in order to adjust the conditions of the detector to the different response of the peaks.

2.3. Conditions

2.3.1. Sample preparation

All the samples were passed through Millex-GV^{13}, 0.22 μm filter (Millipore), which did not retain any of these compounds. The samples (wine, juice and beer) were injected without sample preparation or after dilution up to four times with water. Aliquots of 20 μl were injected manually.

2.3.2. Columns

The analytical column was a Hypersil column ODS C_{18}, (200×2.1 mm), 5 μm particle size (Hewlett-Packard), with pre-column (20×2.1 mm) of the same stationary phase.

Other columns tested were Purospher RP-18, 250×4.6 mm, 5 μm particle size (E. Merck, Darmstadt, Germany); Purospher RP-18 endcapped, 250×4.6 mm, 5 μm particle size (E. Merck) and a C_8 column, Hypersil WP300-10, 250×4.6 mm, 5 μm particle size (HPLC Technology, Macclesfield, UK).

2.3.3. Mobile phase

The following solvents were used: solvent A, 0.05 *M* buffer NaH_2PO_4 at pH=3.0 with H_3PO_4 and solvent B, acetonitrile. The mobile phase was passed through a membrane (0.45 μm) and degassed with He. The linear gradient elution profile was as follows: 0 min, 95% A, 5% B; min 8, 75% A, 25% B, min 12, 95% A, 5% B, with a postime of 3 min and with a constant flow-rate of 0.6 ml/min.

2.4. Sensorial analysis

The average intensity of the ''sunlight flavor'' was estimated with the method of Pichler [6] by a panel formed by eight tasters (researchers and technicians). Each one of the 26 different beverages (wine, beer and fruit juice) was prepared with and without the addition of RF (1 mg/l) and the two sets of samples were subsequently exposed to light. A 100-ml volume of sample was poured into a glass column and exposed to direct artificial light (150 W clear tungsten bulb E27-ES, distance from the source 30 cm, for 48 h). Pairs of each one of the 26 samples were presented to each panelist in the same glass column after the exposure to direct artificial light. Panelists were trained beforehand to perceive the ''off-flavor'' aroma. Panelists rated the intensity of the off-flavor aroma on a three-point category scale (0=not present; 1=perceptible; 2=intense). The average of the evaluations of the eight tasters gave a numerical value variable from 0 to 2, proportional to the intensity of the sunlight flavor.

3. Results and discussion

The three principal natural forms of vitamin B_2 (FAD, FMN and RF) can be satisfactorily separated from wine, fruit juice and beer samples (Fig. 1). In the preliminary phase of method development we considered different columns, detectors and mobile phases, taking into account the polarity, ionic strength and pH. Preliminary tests were done in an HPLC system (HP1090M) equipped with diode-array detection, monitoring B_2 vitamins at 266 and 440 nm.

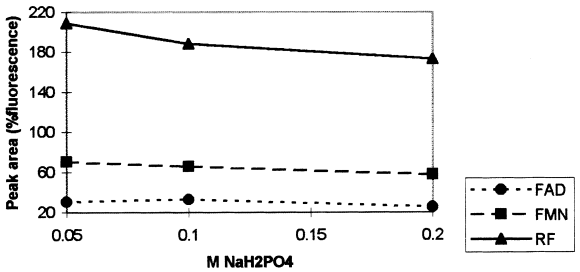

Fig. 2. Changes in peak area (% of fluorescence) of standard solutions with variation in the molarity of the buffer in the mobile phase.

3.1. Column and mobile phase

Preliminary trials were made comparing the narrow bore Hypersil ODS C_{18} column proposed in the present method and three other reversed-phase column with the same particle size: the Purospher RP-18, the Purospher RP-18 endcapped and the Hypersil C_8 WP. Preliminary experiments were made also with different mobile phases, i.e., CH_3CN or CH_3OH combined with phosphate buffer or dilute (0.01%) perchloric acid.

In spite of different resolution, it was always possible to find proper conditions of elution for RF with each one of the different columns, by optimizing the mobile phases. On the contrary, FAD and FMN were partially irreversibly adsorbed in three of the columns. For this reason we chose to further develop the method with the Hypersil C_{18} column, which was the only one who allowed us to quantitatively elute all three analytes.

Strong interactions between analytes and column, leading to asymmetrical and too wide peaks, were still evidenced when MeOH was used. In particular, tailed peaks were observed for FAD, and doubled peaks for RF, when working with perchloric acid–CH_3OH gradients. The RF response was improved

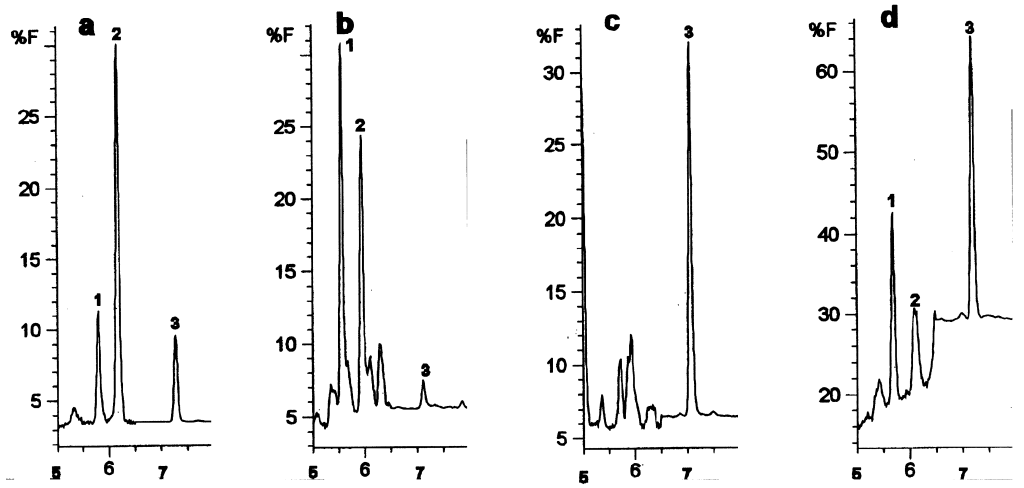

Fig. 1. Chromatographic analysis of a standard solution (a), an orange juice (b), a white wine (c) and a wheat beer (d). 1=FAD; 2=FMN; 3=RF. Time scale in min; F=fluorescence.

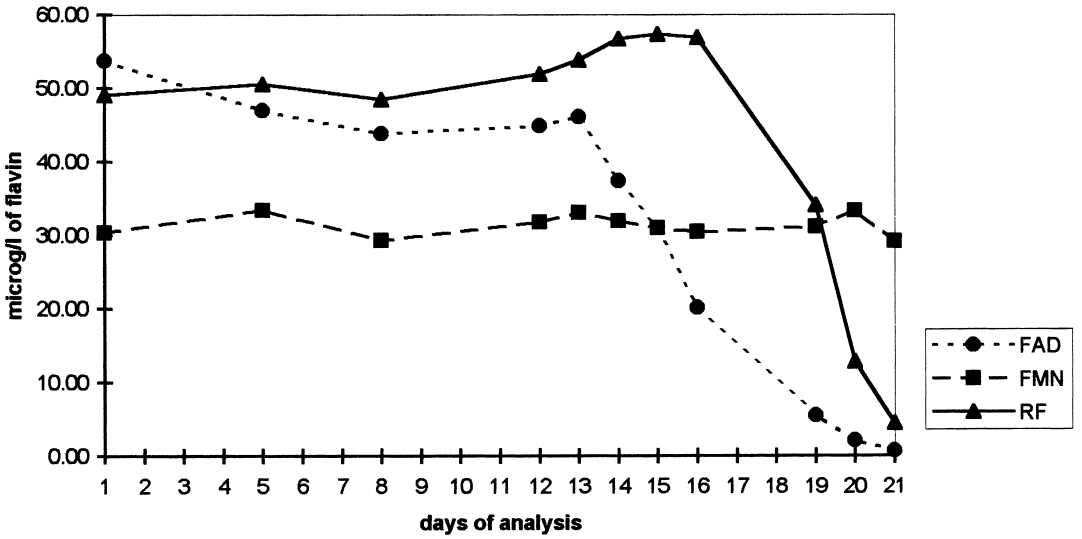

Fig. 3. Changes in the values of RF, FAD and FMN of a mixture of standard, diluted in water, injected over 21 days.

replacing methanol with acetonitrile, and – in agreement with the literature – the response of FAD and FMN was improved replacing diluted perchloric acid solutions with a phosphate buffer.

The fluorescence intensity of flavins is pH dependent [36,37]. FAD has about 20% lower fluorescence efficiency than FMN and RF [38,39]. FAD has maximum fluorescence at pH 2.7–3.1, and RF and FMN show the same intensity of fluorescence over the pH range from 3.7 to 7.5 [40,41]. A mobile phase of pH=3.0 was chosen to provide the maximum fluorescence of the FAD and 85% of the maximum fluorescence of the other two flavins.

The ionic strength of the mobile phase was tested with buffers of different molarity (Fig. 2). The intensity of fluorescence is, in general, inversely proportional to the molarity of the buffer. We chose to work with the low molarity taking also into account the practical negative consequences of working with concentrated buffers.

3.2. Peak identification

Peaks monitored by fluorescence detection were identified by their retention times in comparison with external standards and by standard additions. Their UV–Vis spectra, obtained by injecting in the same chromatographic conditions in an HPLC system equipped with diode-array detection, were also compared.

The peak of RF is a major symmetrical isolated peak in all beverages tested (Fig. 1). In the first part of the chromatogram, close to FAD and FMN, there are always other fluorescent peaks, which make the identification and quantification of small amounts of FAD and FMN less certain. In particular, an unknown peak often occurs, especially in beers and wines, located on the tail of FMN, with an area corresponding to that of a few μg/l of FMN, which makes it difficult to quantify quantities close to the limit of quantitation (LOQ) of this compound in beer and wine. Taking into account this matrix effect, a limit of detection (LOD) approximately equal or slightly higher than LOQ computed on the standard solutions could be suggested for this compound in beer and wine. Sometimes the method of addition of a known amount of standard is required for the correct identification of the peak.

3.3. Peak quantitation

The external standard method was used to measure the concentration. Calibration curves (standard area in % of fluorescence vs. concentration in μg/l) were

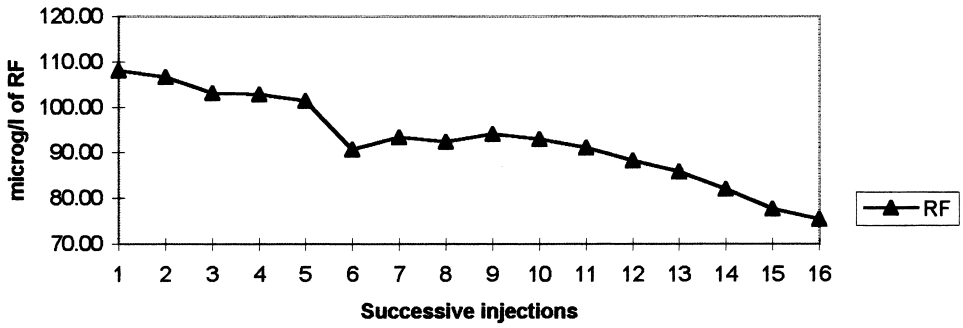

Fig. 4. Changes in the content of RF of an opened wine bottle during successive injections (each 15 min, the HPLC program time) on the same day.

performed in duplicate for each compound (RF, FAD and FMN) over the range of concentration observed in beverages (juices, beers and wines).

3.4. Stability of the standards and samples

To check the stability of the standards, a solution containing known amounts of FAD, FMN and RF in double distilled water was stored in the refrigerator (4°C, dark) and injected on 21 different days (Fig. 3). The standard solution is not stable, since FAD is partially hydrolyzed to RF. For prolonged storage, first FAD and then RF are totally degraded. Only FMN is stable. This demonstrates the necessity to prepare the standard solution just before calibration.

An important observation was noted by sequential repeated injection from the same bottle of white wine, under repeatability conditions. This sample contained RF, while FAD and FMN were only present in traces below the LOQ. The variation of results of repeated analyse on the same day (each 15 min, the HPLC program time), once the bottle has been opened, is shown in Fig. 4. It is clear that the contact with oxygen was causing a continuous depletion of the RF, strongly suggesting the necessity to carry on the determination in a new bottle, which can be opened only at the moment of the analysis.

3.5. Method validation

3.5.1. Selectivity

Excitation and emission spectra of the standards were obtained with the stop-flow technique. To improve the selectivity, each compound was monitored to find the best combination of wavelengths. Excitation and emission wavelengths were set at 265 and 525 nm, respectively, operating with one slit of 2×2 mm on the excitation side and two 4×4 mm slits on the emission side. The cut-off filter was selected at a wavelength close to the λ of maximal emission (500 nm).

Table 1
Average and repeatability R.S.D. (%) of the retention time and concentration of FAD, FMN and RF (n=12)

Compound	t_R (min) average	Repeatability R.S.D. (%)	(μg/l) average	Repeatability R.S.D. (%)
FAD[a]	5.55	0.29	164.40	2.69
FMN[a]	5.94	0.32	49.96	3.06
RF[b]	7.12	0.70	119.83	1.35

[a] Calculated in a diluted orange juice (1:4).
[b] Calculated in a white wine sample.

3.5.2. Precision under repeat conditions

The dispersion of the distribution of test results under repeatability conditions [43] was estimated for the retention times and for the determination of concentration of these three compounds. Results were obtained with 12 analyses of the same sample: an orange juice for FAD and FMN, and a white wine (Pinot grigio) for RF (Table 1). The repeatability relative standard deviation (R.S.D.) of the retention time was below or equal to 0.7%, while the repeatability R.S.D. of concentrations was better for RF than for FAD and FMN, being in every case lower than 3.1%. The repeatability R.S.D.s were lower than those acceptable according to the criteria established by Horwitz for intra-laboratory analysis [42].

3.5.3. Linearity

The linearity of standard curves (Table 2) was expressed in terms of the correlation coefficient (r), from plots of the integrated peak area (% of fluorescence) vs. concentration of the standard (μg/l). These equations were obtained over a wide concentration range, according to the levels of these compounds found in beverages (wine, juice and beer). A linear equation was found, with satisfactory linearity ($r>0.99$) and intercept fairly close to zero.

3.5.4. Sensitivity

The LOD was calculated by repeated injections of diluted solutions, at a signal-to-noise ratio (S/N) of 3, and the LOQ was calculated at an S/N of 10 for all three compounds. These results are shown in Table 3.

3.6. Sample results

In agreement with the assumption of Moll and Charalambous [19], the samples of wines that we analyzed had much lower concentration of FAD and FMN than RF; they were usually below the LOQ of the method (Table 4). Such low concentrations are certainly not important either from the nutritional or from the technological point of view of wine producers. On the basis of these results, it can be observed that for routine analysis of wine, methods assessing only RF can be successfully applied. On the other hand, the juices studied had higher concentrations of FAD than RF (Table 4) except for the tropical juice, which had a declared addition of RF, while in beer samples, small amounts of FAD were always present. For these beverages a method allowing the quantification of all three B_2 vitamins is strongly suggested. Table 4 reports also the estimate of the average intensity of the sunlight flavor after the exposure of samples to light. It is clear that in some of the samples (wines, beers and juices) the defect can be produced, and it is confirmed by comparison of scores after addition of RF (1 mg/l) that a low concentration of the photosensitizer is a limiting factor for the occurrence of this off-flavor.

Table 3
Sensitivity of the HPLC method

Compound	LOD (μg/l)	LOQ (μg/l)
FAD	1.97	6.57
FMN	0.85	2.80
RF	0.49	1.72

Acknowledgements

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Table 2
Linearity of the HPLC method

Compound	Range (μg/l)	Calibration equation	Correlation coefficient
FAD	2.00–16.64	$y=0.400x-0.861$	0.997
FMN	3.04–16.46	$y=0.174x-0.486$	0.999
RF	11.12–222.46	$y=1.707x-1.061$	0.999

y=Concentration (μg/l); x=peak area.

Table 4
FAD, FMN and RF concentration (μg/l) for wines, beers and fruit juices and the estimate of the average intensity of the defect ''sunlight flavor'' after exposure to light of samples

	Flavin content			Sensorial values	
	FAD (μg/l)	FMN (μg/l)	RF (μg/l)	Control	Sample+RF
Pinot grigio wines					
Clone					
SMA 505 (Istituto Agrario S. Michele a/A, Italy), 1996	n.d.	n.q.	85.5	0.38	1.88
SMA 514 (Istituto Agrario S. Michele a/A, Italy), 1996	n.d.	n.d.	94.8	0.38	1.88
GM 1 (Forschunsanstalt Geisenheim, Germany), 1996	n.d.	n.q.	63.2	0.43	2.00
GM 2 (Forschunsanstalt Geisenheim, Germany), 1996	n.d.	n.q.	77.2	0.43	2.00
R 6 (Vivai Cooperativi Rauscedo, Italy), 1996	n.d.	n.q.	56.4	0.43	1.86
49-207 Fr (Staatliches Weinbauinstitut Freiburg, Germany), 1996	n.d.	n.d.	64.6	0.71	2.00
INRA-CV 52 (INRA, France), 1996	n.d.	n.q.	69.1	0.29	2.00
INRA-CV 53 (INRA, France), 1996	n.d.	n.q.	77.7	0.29	2.00
Vineyard (Vallagarina, Trentino, Italy)					
Bagolè, 1996	n.d.	n.d.	89.9	0.63	1.75
Palazzina, 1996	n.d.	n.d.	114.9	0.88	1.88
Acquaviva PR, 1996	n.d.	n.q.	104.5	1.00	1.88
Sabbioni, 1996	n.d.	n.d.	106.4	0.25	1.75
Producer					
Gaierhof 1995	n.q.	3.3	67.4	0.22	2.00
Girelli 1996	n.d.	n.q.	44.3	0.11	1.11
MezzaCorona 1995	n.d.	2.9	83.3	0.78	1.89
Lagaria 1994	n.d.	n.q.	105.8	1.00	2.00
Beers					
Heineken	45.8	n.d.	291.7	0.29	0.57
Forst Premium	19.3	n.d.	235.8	0.14	1.14
Moretti	23.2	n.d.	169.0	0.29	1.29
Franziskaner Hefe-Weissbier	45.4	8.1	272.3	0.43	1.86
Maisel's Weisse	65.2	n.d.	398.9	0.43	1.43
Forst Sixtus	50.2	n.d.	507.9	0.29	0.71
Fruit juices					
Pineapple	104.3	17.3	68.3	0.14	1.89
Orange	171.8	54.4	21.7	0.11	1.88
Grapefruit	100.8	39.4	39.2	0.14	1.75
Tropical (addition of RF declared)	131.0	36.7	3493	2.00	2.00

n.d.=Not detected; n.q.=not quantified.

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