Determination of Vitamin D in Fortified and Nonfortified Milk Powder and Infant Formula Using a Specific Radioassay after Purification by High-Performance Liquid Chromatography

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A reliable and sensitive radioassay is described for the determination of vitamin D in milk powder and infant formula. After saponification of the sample interfering compounds like sterols are removed by digitonin precipitation and chromatography on small columns packed with alumina. [^{3}H]Vitamin D is added to the sample as an internal standard, to correct for losses due to previtamin D formation, as well as other procedural losses. Vitamin D is quantitated by a competitive protein binding (CPB) assay after final cleanup of the extract on a straight-phase HPLC column. Diluted sheep serum is used as the source of the binding protein, having equal affinity for both vitamins D_2 and D_3. With this HPLC–CPB method vitamin D can be determined with high specificity in concentrations as low as 0.1 ng/tube (ca. 0.01 IE/g). Recovery was between 90 and 110%. The coefficients of variation were 2.4 (within run) and 7.5 (between run), respectively.

INTRODUCTION

Vitamin D is essential as a micronutrient in human nutrition for certain groups and under special circumstances only, i.e. when vitamin D needs cannot be met by endogenous production and only be covered by oral intake. Among the groups exposed to the risk of developing vitamin D deficiency are infants in the suckling period.

Cow's milk, but probably also human milk is a rather poor source of vitamin D; contents have been reported to vary between 5 and 40 IU/L. Earlier reports on the antirachitic activity of the water-soluble whey fraction (LeBoulch et al., 1974) could be confirmed neither with a biological rat assay (Leerbeck and Sondergaard, 1980) nor with sophisticated analytical techniques (Hollis et al., 1981a). In many countries milk and milk products are therefore fortified at a level of about 4 IU/mL.

Determination of vitamin D at these low levels is still an analytical problem. Quantification of vitamin D using spectrophotometric procedures or gas–liquid chromatography is always hampered by insensitivity and nonspecificity, requiring lengthy and tedious cleanup procedures and large sample volumes (Parrish, 1979). The application of high-performance liquid chromatography (HPLC) led to major advances in vitamin D analysis in the last few years.

Several HPLC procedures for analysis of vitamin D in completely or partially skimmed milk as well as soy-based infant formulas have been described (Barnett et al., 1980; Borsje et al., 1982; Cohen and Wakeford, 1980; Henderson and Wickroski, 1978; Muniz et al., 1982; Okano et al., 1981; Thompson et al., 1977, 1982).

Basically, all reported methods include saponification or direct extraction as well as one or more (chromatographic) purification steps. The vitamin D content is then quantified by measurement of the UV absorption at 254 or 265 nm, after HPLC on an analytical, straight-phase (SP), or reversed-phase (RP) column.

Some authors claim that in the analysis of nonfat or skimmed milk the saponification step can be omitted and only a limited cleanup of the extract is necessary (Barnett et al., 1980; Cohen and Wakeford, 1980). Chromatography of crude lipid extracts may however lead to distorted vitamin D peaks due to the presence of sterols, to reduced column life, and especially with RP systems to losses of vitamin D as a result of incomplete solubilization of the residue in the solvent.

Quantification by measuring the UV absorbance will always limit the specificity and the sensitivity of HPLC–UV methods. Despite extensive purification inaccurate results cannot be excluded (Hollis, 1983).

The possibility of quantifying vitamin D by radioassay, based on the competitive binding of vitamin D with a specific-protein vitamin D binding globulin, present in rat serum was first described by Haddad and Chyu (1971). In principle the competitive protein binding (CPB) assay is more sensitive and more specific than the existing UV methods. Hollis et al. (1981B, 1983) applied this technique for determining vitamin D in nonfortified human and bovine liquid milk. However, in their method the saponification step is omitted and an extensive prepurification procedure is necessary, including two precipitation steps, an alkaline back-wash step, silica SEP-PAK chromatography, and two successive preparative HPLC runs.

In this report we describe a simple radioassay for the determination of vitamin D in dried milk powders and infant formulas that requires only one precipitation step and one HPLC run. After saponification the sample extract is subjected to digitonin precipitation and is further purified by consecutive chromatography on aluminum oxide and SP HPLC column.

MATERIALS AND METHODS

HPLC Apparatus. *System*: HPLC was performed on an isocratic system incorporating a Kipp Analytica 9208 constant-flow pump (Kipp Analytica, Delft, The Netherlands), a Rheodyne type 9125 injection valve equipped with a 300-μL sample loop, and a Perkin-Elmer LC 75 variable-wavelength absorbance spectrophotometer. A Hyperchrome stainless-steel column, 250 × 4.6 mm i.d. (Salm and Kipp, Breukelen, The Netherlands), was home packed with Polygosil Si 60-5 (Machery-Nagel, Duren, Switzerland; Catalog No. 71101) by the balanced-density slurry technique, using a Haskel pump type MCP 110 (Ammann Technik, Stuttgart, FRG). A guard column of 2 cm with the same packing material was used.

Mobile Phase for HPLC: 0.5% (v/v) isopropyl alcohol in *n*-hexane.

Aluminum Oxide Columns: neutral alumina (Baker Chemicals) dried overnight by heating in a furnace at 600

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0021-8561/86/1434-0264$01.50/0 © 1986 American Chemical Society

°C. After cooling, water was added up to 7% by weight with frequent shaking in a tightly closed glass jar. Deactivated alumina was stored in a tightly closed jar in a desiccator. Every time the alumina was used, the activity was tested by adding a slurry of alumina in hexane to 20 × 0.7 cm glass column (Bio-Rad Laboratories, Richmond, CA). After the column had settled, ca. 10000 dpm of [^{3}H]vitamin D in hexane was applied onto the column, which was eluted with (a) 30 mL of hexane, (b) 70 mL of hexane–ether (92:8, v/v), and (c) 30 mL of hexane–ether (60:40, v/v), respectively. The radioactivity should be completely recovered in the last 20 mL of eluate.

Reagents. *General Reagents*: glass-distilled grade benzene (Rathburn Chemicals Ltd., Scotland); 1.5% (w/v) digitonin in methanol.

Working Buffer: 0.04 M barbital buffer, pH 8.6, containing 0.2% (w/v) bovine serum albumin (BSA), 0.154 M NaCl, and 0.05 M sodium azide.

Vitamin D Tracer Solution: [1,2(*n*)-^{3}H]vitamin D_3 (Amersham International Ltd.; Catalog No. TRK 346), sp act. 15–25 Ci/mmol; purified on SP HPLC as described under procedures. The vitamin D containing fraction was evaporated under nitrogen, and the residue was redissolved in 96% (v/v) ethanol (12 ng/mL; 0.78 μCi/mL).

Vitamin D_3 Standard Solution: crystalline vitamin D_3 (E. Merck, AG, Darmstadt, FRG; Catalog No. 500076); accurately weighed into an amber volumetric flask and dissolved in ethanol. The exact concentration was checked by measuring the absorbance at 265 nm. Directly after spectrophotometric calibration, 0.1% BHT was added. The working standard solution contained 300 IU/mL.

25-Hydroxyvitamin D_3 Tracer Solution: [26,27-^{3}H]-25-hydroxyvitamin D_3 (Amersham International Ltd.; Catalog No. TRK 655), sp act. 150–160 Ci/mmol. After the solvent was evaporated, the residue was redissolved in ethanol. Final dilution was 30 pg/10 μL (ca. 26000 dpm). The radiochemical purity was periodically checked by SP HPLC analysis.

Vitamin D Binding Protein Solution: sheep serum diluted 1:25000 in working buffer. The final dilution depended on the affinity of the binder preparation; the initial binding (B_0/T) in the assay (see below) should be between 35 and 45%.

DCC Solution: 100 mL of working buffer, 3 g of Norit A, and 0.3 g of dextran T70 (Pharmacia, Sweden) added and thoroughly mixed. The dextran-coated charcoal (DCC) solution was stored in the refrigerator.

Procedures. *Saponification and Extraction.* About 10 g of powdered sample was accurately weighed into an amber saponification flask to which 50 mL of ethanol, 20 mL of a freshly prepared 20% (w/v) sodium ascorbate solution, 12.5 mL of 50% (w/w) aqueous KOH solution, and 20 μL of the [^{3}H]vitamin D_3 solution were added, respectively. The saponification mixture was refluxed for 45 min on a steam bath. After cooling, 100 mL of benzene was added and the mixture was vigorously shaken for about 3 min. After separation of the phases, the upper layer (benzene–methanol) was transferred to a 250 mL separatory funnel and washed two times with 50 mL of 5% (w/v) aqueous KOH solution. The benzene layer was then washed at least four times with 50 mL of 0.9% (w/v) NaCl until neutral to phenolphthalein. The organic phase was dried by adding small strips of filter paper to the separation funnel. The benzene layer was transferred into a 250-mL boiling flask and evaporated under reduced pressure at 37 °C. The residue was transferred into a brown centrifuge tube by using a small aliquot of petroleum ether and evaporated under a stream of nitrogen.

Digitonin Precipitation. After evaporation of the petroleum ether the residue was redissolved in 5 mL of 1.5% digitonin solution in methanol. Digitonin precipitation was carried out as described by Muniz et al. (1982). Briefly, the tube was placed in a water bath (50 °C) for 2 min. After the volume was adjusted to 5 mL with methanol, 0.5 mL of water was added and the tube was placed overnight at −20 °C. After centrifugation, the precipitation was repeated after adjusting the volume (of the precipitate) to 10 mL with methanol. Two milliliters of water was added and the tube frozen at −20 °C for about 5 min. After centrifuging (500*g*, 15 min), the supernatant was extracted with 5 × 12 mL of petroleum ether. After drying, the organic phase was evaporated and the residue dissolved in 0.1 mL of hexane.

Prepurification of the Extracts Using Aluminum Oxide and HPLC. Columns (20 cm) packed with alumina were prepared, and the sample extract was transferred to the column. The tube containing the sample was rinsed twice with 0.1 mL of hexane, and the rinses were transferred to the column. The column was eluted as described above. After evaporation of the solvent under nitrogen, the residue was redissolved in 250 μL of HPLC mobile phase and totally injected onto the silica column by using a 300-μL sample loop. The retention time for vitamin D was established for every run by injection of vitamin D standard solution (ca. 300 IU/mL) and monitoring the UV absorbance at 265 nm (retention time about 15 min). The fraction eluting between 2 min before and 2 min after elution of the vitamin D (standard) peak was collected. The solvent was evaporated under nitrogen and the residue was redissolved in 1 mL of ethanol.

Determination of Vitamin D Recovery. A 200-μL portion of the ethanol extract was transferred to a liquid scintillation vial (minivials; Baker Chemicals), and 4 mL of scintillation fluid (Aqualuma Plus; Lumac; Catalog No. 1107) was added. The radioactivity was counted. The percentage recovery was calculated by dividing the radioactivity (dpm) added to the saponification flask by the radioactivity counted in the residue after HPLC.

Quantification of Vitamin D by Competitive Protein Binding Assay. Every sample was assayed in duplicate directly and after a different dilution with ethanol, i.e. 1:2, 1:4, and 1:8, respectively. Standard curves were constructed in the range of 0.5–25 ng/tube vitamin D_3 in 50 μL of ethanol. A 50-μL portion of the undiluted and of the diluted extract (standard) was transferred each to an incubation tube (12 × 75 mm Pyrex borosilicate culture tubes; Corning Glass Works). [^{3}H]-25-hydroxyvitamin D3 (10 μL) solution (30 pg/10 μL) and 500 μL of binder solution in barbital buffer were added, respectively. After shaking, the tubes were incubated at 4 °C for 48 h. For each sample dilution and standard point the nonspecific binding was determined by running a series of tubes in parallel containing 50 μL of sample or standard, 10 μL of 25-hydroxyvitamin D tracer solution, and 500 μL of barbital working buffer, respectively. After incubation, 200 μL of cold DCC solution was added to each tube. After incubation at 4 °C for 30 min the tubes were centrifuged (15 min at 300*g*). The supernatant (500 μL) was transferred into a liquid scintillation vial, and after addition of 4 mL of scintillation fluid the radioactivity was counted.

Calculation of Results. The vitamin D potency in the sample was given as

$$X(20)(100)/25RW = \text{IU/g}$$

where X = amount of vitamin D (ng) read from standard curve, R = percentage recovery of the radioactivity added, 20 = sample dilution factor, 25 = calculation factor from

Table I. Recovery of Added Vitamin D_3, 25-Hydroxyvitamin D_3 (25-OHD), Cholesterol (CHOL), and Palmitic Acid (PA) by the Proposed Purification Procedure[a]

	rec in vitamin D fractn							
	+ digitonin prec				− digitonin prec			
	D_3	25-OHD	CHOL	PA	D_3	25-OHD	CHOL	PA
saponificn, extrctn	90	90	85	0.05	90	90	85	0.05
alum oxide col	70	0.1	0.9		76	0.1	43	
HPLC	54	>0.01	>0.01		58	>0.01	1.4	

[a] To 10 g of homogenized modified milk powder (fat content 27.4% w/w) 150 000 dpm of [^{3}H]vitamin D_3 (S.A. 25 Ci/mmol), 100 000 dpm of [^{3}H]-25-OHD (S.A. 158 Ci/mmol), 150 000 dpm of [^{3}H]-PA (S.A. 25 Ci/mmol), and 115 000 dpm of [^{14}C]-CHOL (S.A. 55 Ci/mmol) were added respectively. The percentage of residual radioactivity in the vitamin D containing fraction after the various procedures is given.

nanograms to international units, and W = weight of the sample (g).

RESULTS AND DISCUSSION

Saponification and Extraction. Saponification of the samples was found to be necessary to ensure complete extraction of the vitamin, especially when large amounts of lipids are present as in dried whole milk or partially skimmed milk. Saponification is also necessary for samples fortified with coated forms of vitamin D.

Direct extraction without saponification (Cohen and Wakeford, 1980) or enzymic hydrolysis (Barnett et al., 1980) resulted in incomplete extraction and caused problems in the consecutive chromatographic steps due to the presence of residual lipids in the extract. Recovery of added vitamin D after saponification and extraction was found to be >90% (Table I). As shown in Table I, free fatty acids, which may interfere with the CPB assay, are effectively removed during saponification and extraction. For extraction of the nonsaponifiable matter benzene is used, as originally described by Mulder (1957), in spite of its highly carcinogenic properties, which requires working in a fume hood. With other solvents like diisopropyl ether, pentane, or hexane, acceptable recoveries were also obtained, but all caused residual effects in the CPB analysis leading to erroneous results (see below).

Digitonin Precipitation, Cleanup by Aluminum Oxide, and High-Pressure Liquid Chromatography. The nonsaponifiable lipid fraction obtained after saponification and extraction may still contain sterols and other lipids that strongly interfere with the CPB assay of vitamin D, resulting in lower specific binding and higher nonspecific binding (NSB). Chromatography on small aluminum oxide columns was found to be a powerful cleanup procedure.

With this step 25-hydroxyvitamin D (25-OHD) is also effectively removed. Because 25-OHD has a much higher affinity for binding on the vitamin D binding globulin (DBG) than vitamin D itself, the presence of even small amounts of 25-OHD may lead to gross overestimation of the vitamin D content of the sample.

Also, cholesterol is partly lost in this step; however, about 45% of added radioactively labeled cholesterol was found to be present in the vitamin D fraction after chromatography on aluminum oxide (Table I). A digitonin precipitation step was necessary for the complete removal of cholesterol. Lipid reduction can also be achieved by methanol and/or ether precipitation, although we sometimes experienced considerable losses of vitamin D (>25%) using these procedures.

Improvement of the specificity of the CPB assay and a significant reduction in the NSB (see below) was obtained after further cleanup of the extract, using HPLC.

Preparative HPLC has been described with both straight-phase (SP) (Thompson et al., 1982; Hollis et al., 1981) as well as reversed-phase (RP) columns (Okano et al., 1981; Borsje et al., 1982). With SP HPLC *cis*-vitamin D is separated from previtamin D, while vitamins D_2 and D_3 are coeluted. On RP HPLC columns D_2 and D_3 are separated while *cis*-vitamin D and previtamin D coelute.

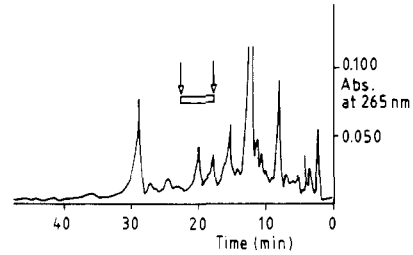

Figure 1. HPLC profile of the unsaponifiable matter of a sample of homogenized modified milk after digitonin precipitation and chromatography on aluminum oxide. The fraction collected for CPB analysis is indicated.

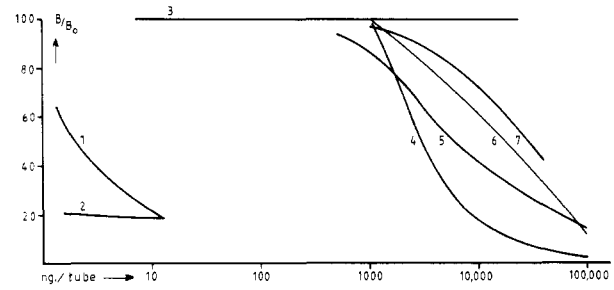

Figure 2. Displacement curves of vitamins D_2 and D_3 and related compounds in the competitive protein binding assay (experimental details in text). Binding is represented as percentage of the ratio B/B_0, where B_0 is the binding of the tracer without added standard: (1) D2, D3; (2) D3 + 5 μg of cholesterol; (3) previtamin D; 5,6-*trans*-vitamin D; (4) cholesterol; (5) 7-dehydrocholesterol; (6) dihydrotachysterol; (7) tachysterol.

For quantification of vitamin D by CPB assay, separation between the pre and cis forms is not necessary, because previtamin D has no affinity for the binder (Figure 2). However, SP rather than RP HPLC allows for correction of the previtamin D formed during saponification and other heating steps. For ^{3}H-labeled and nonradioactive vitamin D the ratio between the pre and cis forms was found to be similar, at room temperature as well as after heating. Addition of [^{3}H]vitamin D to the sample can therefore not only be used for correction of procedural losses but also for losses due to previtamin D formation. Only the radioactivity present in the *cis*-vitamin D fraction is counted and used for calculation of the "overall" recovery.

As illustrated in Figure 1, usually no free vitamin D peak is observed because of the presence of many UV-absorbing substances. The vitamin D fraction collected after SP HPLC can directly be used for quantification in the CPB assay.

Determination of Vitamin D with a Competitive Protein Binding Assay. Quantification of vitamin D is

Table II. Dilution Test for Validation of the CPB Assay for Vitamin D (Read from Standard Curve)

undil extr, ng	dilutn factor 2, ng	dilutn factor 4, ng	dilutn factor 8, ng
9.2	5.1	2.5	1.3
14.2	7.5	4.0	2.0
18.4	10.1	5.4	2.8
>25	>25	17.6	7.8

Table III. Precision of the Method for Determination of Vitamin D in Homogenized Modified Milk Powder

	within run (repeatblty)	between run (reproducblty)
N	5	5
mean, IU/g	4.2	4.0
SD	0.1	0.3
d_{CV}	2.4	7.5

performed by means of a CPB assay, using sheep serum vitamin D binding globulin (DBG), diluted ca. 1:25.000. According to Horst et al. (1981), sheep serum can bind vitamins D_2 and D_3 with equal affinity. With DBG-containing sera from other species, cross-reaction of D_2 and D_3 are different, necessitating separation of both vitamin forms by RP HPLC. However, we have experienced that not all batches of sheep serum show equal affinity for D_2 and D_3 under the conditions described by Horst; careful selection of batches binding serum is therefore essential. When no binder with equal affinity for D_2 and D_3 is available, separation of both vitamers is necessary, e.g. by RP HPLC (Borsje et al., 1982), while vitamins D_2 or D_3 should be used for construction of the standard curve. Previtamin D, 5,6-*trans*-vitamin D_3, tachysterol, and provitamin D (7-dehydrocholesterol) showed no, or a very low, binding with DBG when [^{3}H]-25-OHD was used as the tracer (Figure 2). Comparable displacement curves were reported by Belsey et al. (1974) and Holick et al. (1981). Although cholesterol also showed low cross-reactivity, in the presence of vitamin D a strong interference was observed, i.e. resulting in a distorted standard curve.

For complete solubilization of vitamin D in the assay buffer addition of 0.2% BSA is necessary. Every batch of BSA should be checked on the absence of DBG before use. Even lower blank values can be obtained with Triton X-405 as solubilizer, but its use was discarded, because this compound also reduces the specific binding.

With some solvents problems were encountered because of residue effects in the CPB assay. When standards were dissolved in tubes containing residues of evaporated solvents a shift in the binding curve was sometimes observed, especially with batches of diisopropyl ether, hexane, and pentane.

The effect was minimal with glass-distilled benzene. With every assay reagent blanks were taken throughout the procedure. For every sample three dilutions of the extract were analyzed to check on parallelism and to increase the specificity of the assay. Another advantage of analyzing different dilutions is that a greater range of vitamin D levels is covered. As shown in Table II, linear dilutions were obtained. With some samples nonlinearity was observed when the precipitation step, or one of the chromatographic cleanup procedures, was omitted. The sensitivity of the method, (defined as $B/B_0 = 90\%$), is ca. 0.1 ng/tube, i.e. a minimally detectable vitamin D level of about 0.01 IE/g of sample.

Precision and Accuracy of the Method. In order to determine the precision of the method five replicate portions of one homogenized fortified milk powder were analyzed in one run as well as in five successive assays. The results are shown in Table III. The accuracy of the method was assessed by measuring the recovery of added vitamin D_3 (Table IV), as well as the dependence of sample size (Table V).

Table IV. Recovery of Vitamin D_3 Added to a Homogenized Modified Milk Powder

vit D_3 added, IU/g	found, IU/g	rec, %
none	5.0	
2.0	6.9	95
3.0	7.7	90
4.0	9.4	110
6.0	10.9	98

Table V. Determination of Vitamin D Content of Homogenized Whole Milk Powder Relative to the Amount Analyzed

amt sample, g	found, IU/g	amt sample, g	found, IU/g
5.0	3.3	15.0	3.3
10.0	3.1	20.0	3.0
12.5	2.8		3.1 + 0.2[a]

[a] Mean value.

Table VI. Vitamin D Content (IU/g) of Some Milk Powders and Infant Formulas Analyzed with the HPLC-CPB and the HPLC-UV Methods

sample	label claim	obsd HPLC-CPB	obsd HPLC-UV
1. unfortif whole milk powd (27% fat)	ca. 0.2	0.1	nd[a]
2. fortif whole milk powd (27% fat)	4.5	4.6	na[b]
3. unfortif skimmed milk (0.5% fat)	?	0.01	nd
4. fortif skimmed milk (0.5% fat)	4.5	3.9	3.9
5. Frisolac	4.0	3.6	na
6. Almiron M2	4.0	5.4	5.9
7. Similac	4.0	4.3	5.7
8. IV-25	?	0.6	0.8
9. IV-25 + 4.0 IU/g	4.0	4.7	4.6

[a] nd = not detectable. [b] na = not analyzed.

As can be concluded from these tables precision and accuracy are both adequate.

Application of the Method: Conclusions. The method described here can be applied to a broad variety of milk powders and dairy products, irrespective of the lipid content of the sample. In Table VI results for several fortified and nonfortified milk samples are presented. Some samples were also analyzed by HPLC-UV. With the HPLC-UV method the same extraction and cleanup procedure was used, but after SP HPLC, a RP HPLC column (250 × 4.6 mm Lichrosorb ODS; E. Merck, A. G., FRG; mobile phase methanol-water (95:5)) was used for quantification of vitamin D. As illustrated in Table VI, comparable results were obtained, but the HPLC-CPB method allowed determination at lower levels. The main advantage of this method is the possibility of reanalyzing samples when doubtful results are obtained with the HPLC-UV methods, possibly due to the presence of UV-absorbing compounds coeluting with vitamin D. Overestimation with methods based on UV quantitation has been reported (Hollis, 1983).

Due to the high sensitivity (100 pg absolute) this method can also be applied to samples having a low vitamin D content like unfortified milk products and human milk samples. Other advantages are that previtamin D correction is easily performed and the total vitamin D activity can be measured.

However, the use of radioactive tracers may be a limiting factor in some laboratories. The working time required

for one analysis is comparable with that of HPLC–UV methods. However, it takes about 3 days to produce the results, because of the relatively long incubation time of the CPB assay.

Registry No. Vitamin D, 1406-16-2.

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Received for review May 7, 1985. Revised manuscript received September 16, 1985. Accepted October 9, 1985.

Analysis of Methoxypyrazines in Wines. 1. Development of a Quantitative Procedure

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A new method for the quantification of 2-methoxy-3-isobutylpyrazine (3IBP) from wine was developed. Wine was spiked with both 3IBP and 2-methoxy-3-isopropylpyrazine (IPP) and extracted by steam distillation. The distillate was adsorbed onto a C-18 SEP-PAK cartridge with subsequent methanol elution. This concentration step was followed by analysis of the eluant by reversed-phase high-pressure liquid chromatography (HPLC). The recovery for 3IBP was 52.9 ± 7% with a minimum detection level of 1.2 μg/L (assuming 50% recovery). For IPP the recovery was extremely inefficient with only 14 ± 4.7% recovered. As demonstrated herein, light must be excluded from the analysis. After exposure to light for 120 h, aqueous solutions of either methoxypyrazine showed a loss of approximately 28% by photodegradation, whereas no change occurred in samples from which light was excluded.

Pyrazines are found in a large number of foods and other natural products. Most alkylpyrazines are formed by Maillard reactions via Strecker degradation when foods are heated, although some are produced by microorganisms such as *Saccharomyces cerevisiae* in beer and wine (Maga, 1982; Kosuge et al., 1971). These compounds are partly responsible for the pleasant aromas of roasted meats, coffee, cocoa, and cereals (Maga, 1982). Alkoxypyrazines are found in raw vegetables such as peas, bell peppers, potatoes, beets (Murray and Whitfield, 1975), and grapes (Bayonove et al., 1975; Augustyn et al., 1982). These generally "vegetative" or musty, earthy compounds are produced as contaminants in water, milk, and other fluids mainly by *Actinomyces spp.* (Mottram et al., 1984; Gerber, 1979; Morgan, 1976; Miller et al., 1973).

The alkoxypyrazine 2-methoxy-3-isobutylpyrazine (3IBP) was first isolated from bell peppers by using a simultaneous distillation–extraction technique (SDE). This compound, which has the characteristic odor of bell peppers, is a potent odorant; in water it has an odor detection threshold of 2 parts in 10^{12} (Buttery et al., 1969). Bayonove et al. (1975) were the first to tentatively identify 3IBP in Cabernet Sauvignon grapes using a pentane liquid–liquid extraction. However, using a methylene chloride extraction, Slingsby et al. (1980) were unable to detect any 3IBP in a Cabernet Sauvignon wine that had a distinctly vegetative aroma. In addition to 3IBP, 2-methoxy-3-isopropyl- and 2-methoxy-3-*sec*-butylpyrazine were tentatively isolated from Sauvignon blanc grapes by using Freon extraction and headspace techniques (Augustyn et al., 1982). All of these workers only qualitatively analyzed their systems, reporting only whether the compound was detected or not.

Using a headspace technique to determine the approximate levels of the methoxypyrazines, the first quantitative data were collected by Murray and Whitfield (1975) for a variety of vegetables. For this analysis, they claimed "no great accuracy". The only reproducibility or recovery data for analysis of the alkoxypyrazines are those reported by Krasner et al. (1983). Using a closed-loop stripping method followed by gas chromatography, 84 ± 18% IPP and 102 ± 31% 3IBP were recovered from spiked water samples. However, this method is not appropriate for analysis of methoxypyrazines in complex systems such as wine. In

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0021-8561/86/1434-0268$01.50/0 © 1986 American Chemical Society