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Review article

Determination of vitamin D_3 metabolites: state-of-the-art and trends

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Abstract

The steps involved in the methods for the determination of vitamin D_3 metabolites (namely, 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3) mainly in clinical samples are critically reviewed. Sample pretreatment (e.g. deproteinization, saponification, liquid–liquid and liquid–solid extraction, etc.) as a function of both type of sample and detection system, quantitation based on protein saturation and liquid as well as gas chromatography are discussed. The chemical principles on which the methods are based and the derivatization procedures, which facilitate separation and/or detection, are also commented upon. Finally, the future prospects of the research on methods for the determination of these metabolites are outlined.

Keywords: Vitamin D_3; 25-hydroxyvitamin D_3; 1,25-dihydroxyvitamin D_3; 24,25-dihydroxyvitamin D_3; Review

1. Introduction

Vitamin D_3 has scarce biological activity but is converted into biologically active metabolites by oxidation. A first oxidation step occurs in the liver and converts vitamin D_3 into 25-hydroxyvitamin D_3 (25-OH-D_3); the second oxidation reaction takes place in the kidney and converts 25-OH-D_3 either into 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3], the hormonal metabolite of vitamin D_3 endocrine system, and 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3]. Measurements of circulating levels of 25-OH-D_3 is presently useful both in the diagnosis of vitamin D_3 deficiency (a determinant factor of hip fracture in elderly people) and intoxication. The most biologically active metabolite, 1,25-dihydroxyvitamin D_3, is the major hormonal regulator of calcium metabolism and its quantitation is widely used as a means of assessing the

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vitamin D_3 level in humans. Circulating 1,25-$(OH)_2$-D_3 is altered in several pathophysiological states (e.g. parathyroid gland disorders, renal failures, vitamin D-dependent rickets types I and II and sarcoidosis [1,2]).

Simultaneous determinations of these metabolites have been reported and reviewed in the literature [3–5]. The determination of these species in clinical samples has been considered a difficult goal. So far, several reasons justify this assertion: (1) the low concentration of circulating hydroxymetabolites in human fluid; (2) the presence of a number of metabolites which exhibit similar chemical behaviour to one another; (3) the large amount of other related neutral lipids; and (4) the high instability of their chemical structures in the presence of UV light and heat. Increasing the sensitivity and selectivity of the determination of vitamin D_3 metabolites has been the aim of various methods focused on sample cleanup and analyte concentration prior to determination.

In addition to the determination of these species in clinical samples, other types of samples require the analysis of vitamin D_3 and its metabolites. Fig. 1 depicts the areas in which these species have been determined and the frequency of the individual, simultaneous or overall determinations of each of the target compounds. Thus, determination in clinical samples, either serum or plasma, constitutes 73.6%, while 16.5% of the determination corresponds to food and 9.5% to pharmaceutical preparations. As can be seen, vitamins D_2 and D_3 are the vitamins mostly determined in food and pharmaceutical samples; whereas, clinical determinations are mainly devoted to hydroxymetabolites. The sample matrix has a decisive influence on the cleanup and concentration procedures developed prior to quantitation. These steps are minimal in synthetic and pharmaceutical samples, which contain high concentrations of the analytes in very simple matrices. On the other hand, the determination of these compounds in foods and foodstuffs requires

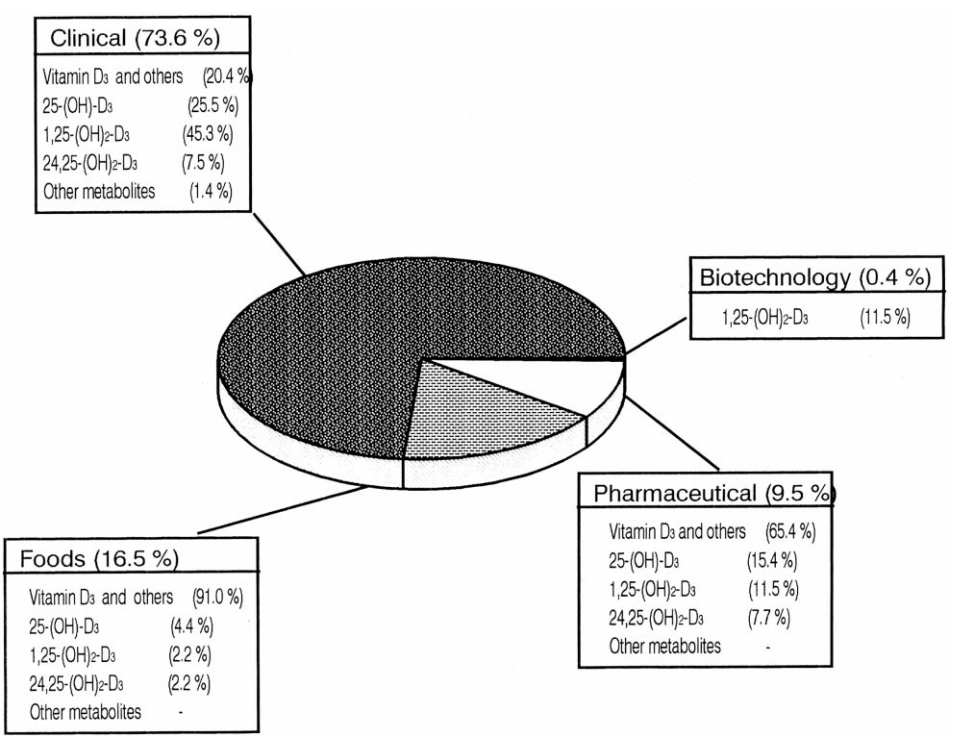

Fig. 1. Application areas for the determination of vitamin D and its metabolites, expressed as percentage.

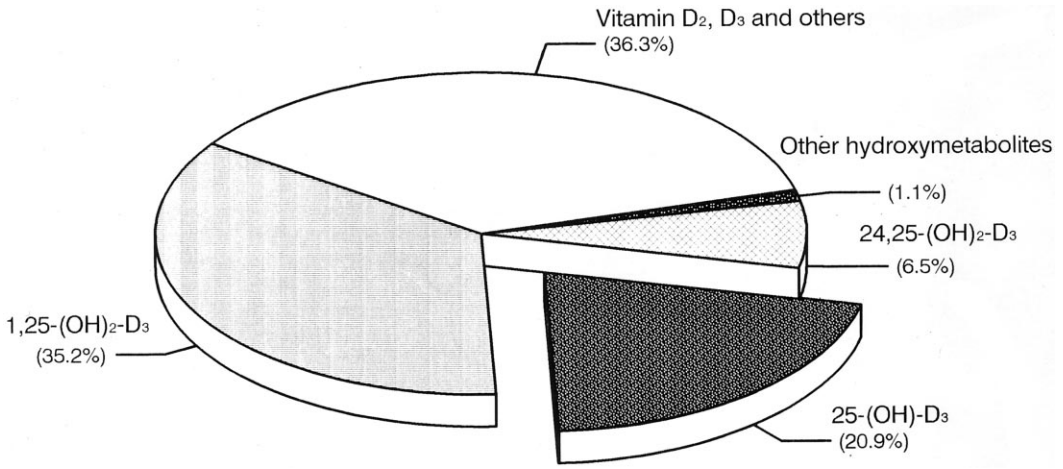

Fig. 2. Percentage of samples in which vitamin D_3 and its metabolites have been determined.

a tedious, time-consuming pretreatment which involves frequently saponification and liquid–liquid extraction. Laborious cleanup and preconcentration steps are also required for the determination of the hydroxymetabolites in clinical samples, particularly for the quantitation of 1,25-$(OH)_2$-D_3, present in serum and plasma at the pg ml^{-1} level.

Fig. 2 depicts the percentage of samples in which vitamin D_3 and its metabolites have been determined. The methods for the determination of vitamin D_3 and its main hydroxymetabolites (namely, 25-OH-D_3, 1,25$(OH)_2$-D_3 and 24,25$(OH)_2$-D_3), including sample pretreatment, derivatization and quantitation, are critically reviewed and the most remarkable trends are outlined herein.

2. Chemical features and derivatization procedures

Vitamin D_3 and its metabolites are 9,10 secosteriods (Fig. 3) which result from cholesterol by opening the A-ring in the C_6 position to yield the cis-triene conjugated molecule [4]. This vitamin occurs in the body as a consequence of the diet and synthesis in the skin from 7-dihydrocholesterol under UV radiation. Vitamin D_3 is transported to the liver by globulin receptor proteins present in blood, where it is enzymatically hydrolysed at the C_{25} level to form 25-OH-D_3. This metabolite is also transported to the kidney by blood, bound to the corresponding receptor protein. In the kidney, 25-OH-D_3 is hydroxylated again at the C_1 or C_{24} level to yield 1,25$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3, respectively [4]. The low chemical reactivity of these compounds is the limiting factor for the development of new analytical methods for their determination. According to their chemical structure, these compounds show molecular absorption maximum at 264 nm ($\varepsilon = 18.300$ l mol^{-1} cm^{-1}), which is the principle of the methods based on UV detection proposed so far. These methods are scarcely selective due to the presence of other compounds which absorb in the UV region. The selectivity is usually enhanced by a chromatographic separation prior to detection.

Recently, several methods based on derivatization reactions, such as dehydration [6–8], cycloaddition [9–12], silanization [13–17] and charge-transfer complex formation [18] have been implemented in pre- or post-column derivatization in either LC or GC.

Dehydration reactions occur by exposure of the analytes to a high temperature. A non-specific dehydration takes place under this condition, producing B-ring cyclation to yield pyro and isopyro isomers. The number of isomeric structures thus produced significantly complicate subsequent individual separation and identification. The first

evidence of cyclation, demonstrated in 1960, constituted a hard limitation for development of GC methods.

Makin et al. reported a selective cyclation with quantitative production of one of the isomers and minor contribution of the others. This selective dehydration was accomplished by applying high temperatures in the presence of small glass-pieces on the top of a GC column. Glass material acted as a catalyst. This effect was later demonstrated using prepacked glass-columns for dehydration prior to the determination of steroid hormones by GC–MS [19]. The same authors developed in 1995 a method for enhancing sensitivity in the determination of vitamin D_2 and D_3 hydroxymetabolites by GC–MS with precolumn dehydration using a prepacked aluminium minicolumn heated at 400°C [20].

Some other methods for the derivatization of vitamin D_3 hydroxymetabolites have been aimed at obtaining more thermostable products by formation of the corresponding isotachysterol. The most remarkable procedures are based on cyclation caused by reaction with a strong dehydrant reagent. Thus, Nai and DeLeon used trifluoroacetic acid to form isotachysterol from vitamin D_3 prior to determination by gas-chromatography [21]. Other common reagents, such as acetyl chloride, penta and heptafluorobutyl chloride and their anhydrides, boron trifluoride, antimonium chloride and hydrochloric gas in chloroform have also been used [22,23]. The production of thermostable compounds has been mainly chosen so as to circumvent the shortcoming associated with the use of GC. The non-specific production of other compounds, such as esters, also yields new isomeric series which complicate the individual isolation of the target analytes.

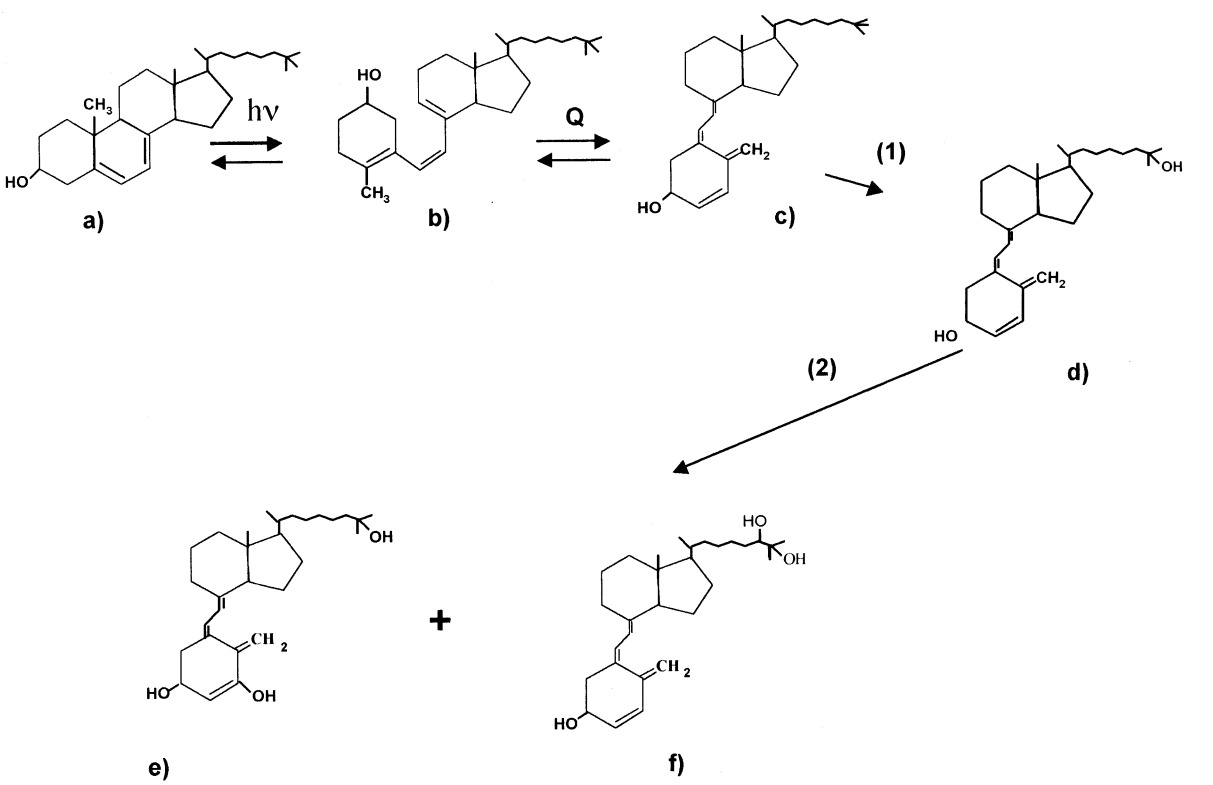

Fig. 3. Basic structure of vitamin D_3 and its metabolites: (a) 7-hydrocholesterol; (b) pre-vitamin D_3; (c) vitamin D_3; (d) 25-hydroxyvitamin D_3; (e) 1,25-dihydroxyvitamin D_3; and (f) 24,25-dihydroxyvitamin D_3.

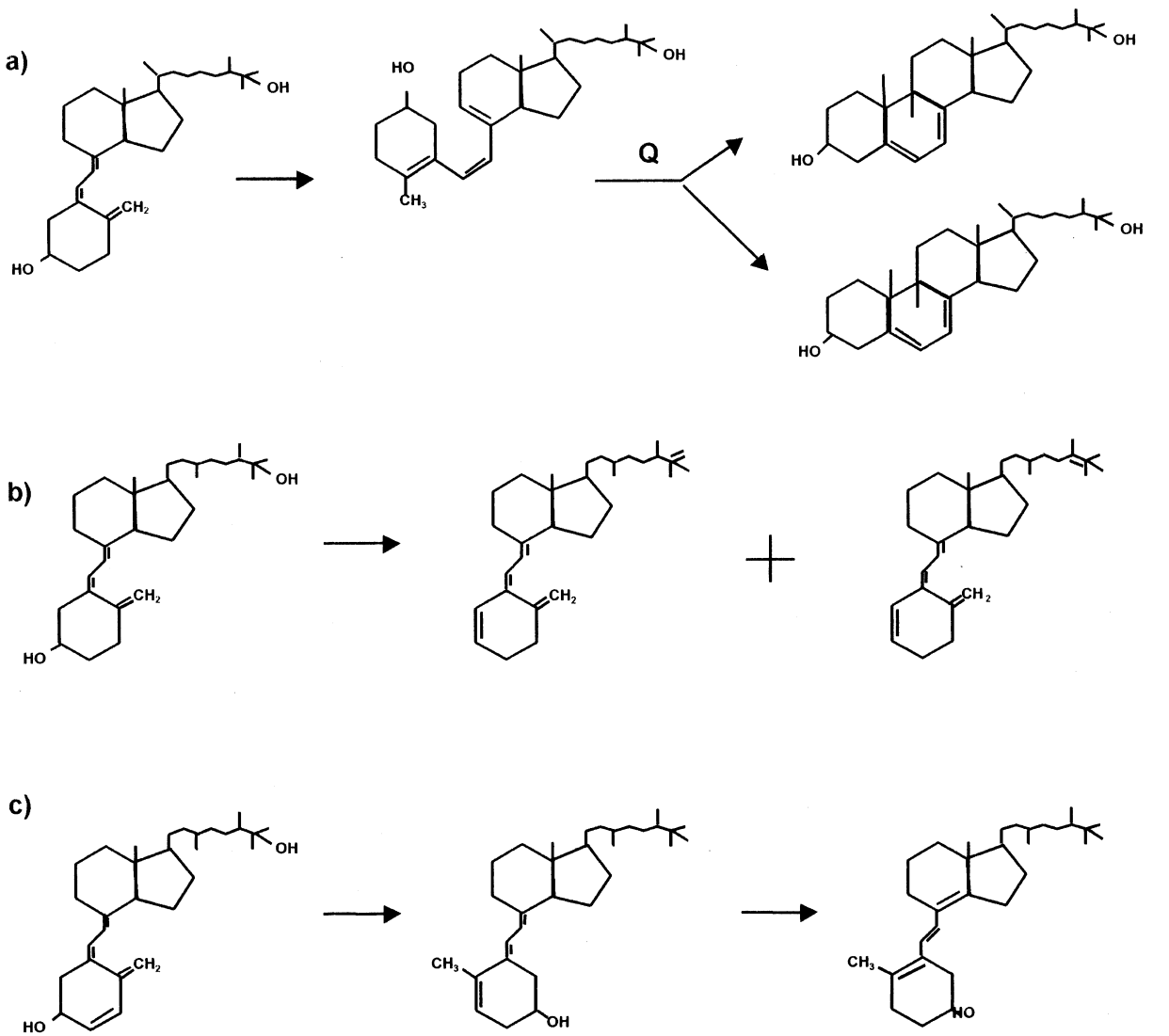

Fig. 4. Derivatization reactions: (a) thermal cyclation of B-ring; (b) selective dehydration by using dehydrating columns at high temperature; and (c) isotachysterols isomerizations by chemical dehydration.

Recently, dehydration based on the use of strong acids, which causes B-ring cyclation in the secosteroid structure has been reported [24]. The isotachysterol formed exhibits an absorption displacement to longer wavelengths, and fluorescence emission in some cases. An enhanced sensitivity is obtained by implementing this type of derivatization; nevertheless, the drastic conditions required make the methods impracticable in routine analysis (Fig. 4).

Diels-Alder cycloaddition reactions constitute another way of enhancing sensitivity and selectivity in the determination of vitamin D_3 and ts hydroxymetabolites as these compounds include in their structure a triene system which is able to produce $2+4$ Diels-Alder cycloaddition at room temperature. With this purpose Cookson's reagent (4-phenyl-1,2,4-triazoline-3,5-dione, known as PTAD) has been used as dienophyle reagent for the derivatization of 25-OH-D_3, 1,25-

$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 [25]. The introduction of a phenyl group in the fourth position of the triazoline-dione (TAD) yields a chemical structure which provides higher UV absorption, thus providing a tool for development of more sensitive methods. The production of TAD reagents endowed with new fluorophore, chromophore and electrophore groups has enlarged the number of sensitive methods for these analytes. However, the presence in the samples of other compounds capable to produce similar products with these reagents has restricted their use. Yamada et al. have developed a new dienophyle reagent by successive condensations of 2-(6,7-dimetoxy-4-methyl-3-oxo-3,4-dihydroxyquinoxaline)-propionic acid to yield 4-[2-(6,7-dimetoxy-4-methyl-3-oxo-3,4-dihydroxyquinoxaline)-2-ethyl]-1,2,4-triazoline-3,5-dione, designated as DMEQ-TAD, which has been used for the development of a new method for the determination of the three main hydroxymetabolites of vitamin D_3 [26]. Also a fluorimetric precolumn derivatization of these metabolites based on a Diels-Alder cycloaddition between a derivative of propionic acid and an azide to yield 6,7-dimetoxy-3-oxo-3,4-dihydroquinoxaline 2-carbonyl azide (DMEQ-CON_3) has been reported by Ohkura et al. [8]. More recent contributions in this field are based on the use of Diels-Alder cycloaddition for pre- or post-column derivatization in HPLC–MS [9–12,27], as well as on PTAD derivatives as electron capturers.

Silanization reactions have been used in order to minimize non-specific adsorption of vitamin D_3 and its metabolites in gas chromatography mainly due to the presence of hydroxyl groups on these molecules. The procedures reported are based on formation of different silylcompounds such as pertrimethylsilyl-eters [28], ter-butyl-dimethylsilyl-eters [29], and *n*-butyl-boronateester-trimethylsilyl-esters [29,30]. Despite the difficulties (namely, long time for development and analyte losses associated with the derivatization step), methods based on these reactions for 1,25-$(OH)_2$-D_3 [19,20,31]; 24,25-$(OH)_2$-D_3 [19,20,31]; 25,26-$(OH)_2$-D_3 [32], and 25-OH-D_3 [19,20] have been reported.

Charge transfer-complex formation with iodide in different media has been the principle on which methods for the determination of vitamin D_3 in pharmaceuticals have been based. Quantitation based on measurement of the third derivative of the absorption spectrum of the complex, is not selective for the analyte; so it has had to be applied to very simple samples [18].

3. Sample pretreatment

Pretreatments based on one or several cleanup/preconcentration steps are mandatory when vitamin D_3 hydroxymetabolites have to be determined in clinical samples. These steps, usually developed manually, have prompted the research to be focused on both the reduction of the time required for their development and the number of steps involved and/or obtaining a cleaner extraction. The aims of these steps are one or several of the following: (1) removal of macromolecules such as proteins and lipoproteins as well as other macromolecules which cause physical interference because of their size; (2) removal of species which chemical and/or structural features similar to those of the analytes; (3) the establishment of patterns for individual fractionation of vitamin D_3 as well as of its metabolites prior to individual separation; and (4) preconcentration of the analytes, usually carried out simultaneously with the separation.

Table 1 lists the sample pretreatments proposed so far, the most interesting of which are as follows:

(A) Deproteinization steps. The removal of proteins and related macromolecules is a common step with serum and plasma samples. This step consists of mixing equal volumes of sample and precipitant reagent such as acetonitrile [19,31–37], ammonium sulphate [38,39], or ethanol [40,41]. This is the only treatment prior to selective quantitation (e.g. RIA).

(B) Pretreatment based on saponification. This step is mainly used with samples containing high contents of lipids such as infant formulae [41,42], enriched milk [43–46], eggs [46], fish oil [47–50] and margarine [51]. It substitutes deproteinization

Table 1
Features of the quantitation methods for vitamin D_3 hydroxymetabolites[a]

Analyte	Sample	Sample pretreatment				Separation technique	Detection system	Ref.
		Deproteination/ Saponification	LLE	SPE	LC			
25-OH	Serum	—	—	—	—	—	RRA/LS	[105]
25-OH	Serum	—	—	—	—	HPLC	UVA	[106]
25-OH	Plasma	—	—	—	—	HPLC	UVA	[107]
25-OH	—	—	—	—	—	—	CPBA/LS	[79]
25-OH	—	—	—	—	—	HPLC	UVA	[108]
25-OH, D_2,D_3	Plasma	—	—	—	—	HPLC	UVA	[109]
25-OH, 1,25-$(OH)_2$	Plasma/serum	—	—	—	—	HPLC	UVA	[110]
25-OH	—	—	—	—	—	HPLC	UVA	[81]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Plasma	—	—	C_{18}	—	—	CPBA/LS	[111]
25-OH, D_2, D_3	Plasma	—	—	—	—	HPLC	UVA	[112]
25-OH, D_2, D_3	—	—	—	—	—	HPLC	UVA	[113]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Renal fluid	—	—	C_{18}	—	HPLC	UVA	[114]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Plasma, urine	ACN	—	—	—	—	—	[32]
25-OH, 1,25-$(OH)_2$	Plasma	ACN	—	C_{18}	Si	—	RIA/LS	[115]
25-OH	—	—	—	C_{18}	—	HPLC	CPBA/UVA	[129]
25-OH	Serum	—	—	—	—	HPLC	EM	[29]
25-OH	Serum	ACN	—	—	—	—	RIA/LS	[31]
25-OH	Serum	—	—	—	—	—	CPBA/RIA/LS	[85]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_2, D_3	Serum	ACN	—	—	—	HPLC	CPBA/RIA/ UVA/LS	[70]
25-OH	Serum	ACN	—	C_{18}	Si	HPLC	CPBA/RIA/LS	[31]
25-OH, D_2, D_3	Plasma	—	$MeOH/CH_2Cl_2$	C_{18}	Si	—	CPBA/UVA	[59]
25-OH, D_3	Plasma	—	$(NH_4)_2$ SO_4/EtOH/K_2HPO_4	LH-20	—	HPLC	UVA	[116]
25-OH, 1,25-$(OH)_2$, D_3	Plasma	—	—	C_{18}/Si	—	HPLC	UVA	[118]
25-OH, D_2, D_3	Plasma	ACN	—	C_{18}	Si	HPLC	UVA	[39]
25-OH, D_2, D_3	Plasma/serum	—	—	C_{18}/Si	Si/C_{18}	HPLC	UVA	[53]
25-OH, D_3	Muscular tissue	—	$CHCl_3$/MeOH	C_{18}	Si/Lipidex/Si	HPLC	UVA	[34]
25-OH, 1,25-$(OH)_2$	Serum	—	—	LH-20	—	—	RIA	[117]
25-OH, 1,25-$(OH)_2$	Serum	ACN	—	Extrelux-1	—	HPLC	UVA	[69]

Table 1 (Continued)

Analyte	Sample	Sample pretreatment				Separation technique	Detection system	Ref.
		Deproteination/ Saponification	LLE	SPE	LC			
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_2, D_3	Plasma	—	$MeOH/CH_2Cl_2$	C_{18}	Lipidex/Si/C_{18}	HPLC	UVA/RIA	[33]
25-OH, D_2, D_3	Plasma/serum	ACN	—	—	—	HPLC	UVA	[87]
25-OH, D_2, D_3	Plasma/serum	—	$MeOH/CH_2Cl_2$	C_{18}	—	HPLC	UVA	[119]
25-OH	Serum	—	—	C_{18}	C_{18}/Si	HPLC	UVA	[37]
25-OH	Serum	—	—	—	—	HPLC	UVA	[73]
25-OH, 1,25-$(OH)_2$	Plasma/serum	—	—	—	—	HPLC	RRA/CPBA/UVA	[120]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$ D_2, D_3	Milk	—	Ethylacetate	Si	—	HPLC	EM	[13]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$ D_2, D_3	Plasma	ACN	—	C_{18}	Si	GC	CPBA/LC	[121]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, 25,26-$(OH)_2$	Infan. Form.	—	$MeOH/CH_2Cl_2$	C_{18}	Si	—	EM	[14]
25-OH, 1,25-$(OH)_2$, 25,26-$(OH)_2$	Plasma	ACN	—	C_{18}/Si	Si	GC	UVA	[122]
25-OH	Plasma	—	—	C_{18}	Sepharon SGX	HPLC	VOL	[44]
25-OH	—	—	—	—	—	—	RIA/LS	[45]
25-OH, D_2, D_3	Serum	—	$MeOH/CH_2Cl_2$ + KOH/CH_2Cl_2 + MeOH	—	Si	HPLC	VOL	[123]
25-OH, D_3	Pharm. Form.	—	—	—	—	TLC/HPLC	UVA(DAD)	[36]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Plasma	ACN	—	C_{18}	—	HPLC	FL	[8]
25-OH, D_3	Serum	—	$MeOH/CH_2Cl_2$	—	—	HPLC	FL	[11]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	—	—	—	—	—	HPLC	FL	[88]
25-OH	Serum	—	—	—	—	HPLC	UVA	[9]
25-OH, 24,25-$(OH)_2$	Plasma	—	$MeOH/CH_2Cl_2$	Si	—	—	RIA/LS	[124]
25-OH	—	—	—	—	—	—	RIA/LS	[125]
25-OH	Serum	ACN	—	—	—	HPLC	UVA	[58]
THF/Ethylacetate	Plasma	—		C_{18}/Si	—	—	RIA/LS	[126]
—	Plasma	—		—	—	—	EM	[10]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	—	—	—	C_{18}	—	HPLC	VOL	[94]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	—	—	—	—	—	HPLC	VOL	[127]
25-OH	—	—	—	—	—	HPLC	EM	[27]

Table 1 (Continued)

Analyte	Sample	Sample pretreatment				Separation technique	Detection system	Ref.
		Deproteination/ Saponification	LLE	SPE	LC			
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	Plasma/serum	—	—	C_{18}	—	HPLC	FL	[89]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	Eggs	EtOH/KOH	—	Si	C_{18}	HPLC	UVA	[128]
25-OH	Pharm. Form.	—	—	—	—	HPLC	EM	[129]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	—	—	—	—	—	HPLC	EM	[12]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$, D_3	Plasma	EtOH	—	C_{18}	LH-20	HPLC	UVA	[24]
25-OH	—	—	—	—	—	HPLC	UVA	[130]
25-OH	—	—	MeOH/CH_2Cl_2	C_{18}/LH-20/C_{18}	—	—	UVA	[5]
25-OH	—	—	MeOH/CH_2Cl_2/ Ethyilacetate/Hexane	Si	—	HPLC	UVA	[7]
25-OH	Plasma	—	Ether/Ethylether	C_{18}	Si	HPLC	UVA	[47]
25-OH, D_3	Fish tins	EtOH/KOH	—	—	—	HPLC	UVA/FL	[15]
25-OH, D_3	Bile	—	—	LH-20	Si	—	RRA/UVA	[131]
25-OH, 1,25-$(OH)_2$	Plasma/serum	ACN	—	—	—	HPLC	UVA	[132]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Plasma/serum	—	Isopropanol/Hexane	C_{18}	—	HPLC	UVA	[65]
25-OH, 1,25-$(OH)_2$, 24,25-$(OH)_2$	Plasma/serum	ACN	—	NH_2	—	HPLC		[67]

[a] The abbreviation of SPE or LC designed the packed column.

and consists of a hot treatment (50–80°C) of a 1:1 sample–0.5 KOH ethanol solution mixture, containing ascorbic acid. Neutralization of the strongly basic solution resulting in the mixture [45,47] follows saponification prior to liquid–liquid extraction. Saponification and subsequent removal of lipids involve losses of both vitamin D_3 and its metabolites by dragging. The analyte losses are evaluated by a recovery study using radioactive isotopes.

(C) Pretreatment based on liquid–liquid extraction. An alternative to the previously commented steps for removal of interfering macromolecules is the implementation of either simple or multiple liquid–liquid extraction steps using solvents of different characteristics. After mass-transfer between the two immiscible phases, these are separated, the organic solvent which contains the analytes is removed usually by evaporation and the concentrated extract is redisolved in the solvent appropriate for subsequent steps. The whole procedure presents several shortcomings, i.e. (a) it is manually developed; (b) it consists of time-consuming steps; and (c) large amounts of the analytes are lost because of partitioning between the two immiscible liquids. Pure solvents and binary or ternary mixtures of polar (52–55) and non polar solvents at different pHs [13,14,42,44–46,56–58] are used depending on the target goal being the overall or selective removal of lipids. Improvements of the separation procedures are accomplished using either non-polar/polar solvent mixtures, which facilitate breaking analyte-transport protein bonds [55]; or low ionic strength/low basicity solvents such as phosphate buffers, which facilitate removal of acidic, low ionized lipids [52]. The most general procedure based on liquid–liquid extraction is that proposed by Bligh and Dyer [54], which has been widely used with small modifications for a long time [55].

(D) Pretreatment based on liquid–solid extraction. This separation step, used since the earliest methods for the quantitation of vitamin D_3 and its metabolites, underwent a remarkable expansion with the commercialization of liquid–solid extraction cartridges, which virtually substituted manual column packing. The improvement of sample pretreatment thus achieved can be summarised as follows: (a) lower amount of neutral lipids in the extract as compared with liquid–liquid extraction; (b) higher protection of the equipment used for subsequent individual separation (HPLC or GC), or by direct quantitation competitive protein binding (CPBA), radioreceptor binding (RRB) or radioimmunoassay (RIA); (c) effective decrease of cost in terms of cartridges and solvents; (d) high preconcentration factors by final analyte elution with small solvent volumes; and (e) availability for selective separation of the analytes based on polarity differences. Nevertheless, the use of this type of pretreatment also involves some drawbacks, such as: (a) the necessity for calibration procedures, which minimize the variability of extraction efficiency due to differences among commercial cartridges; (b) the potential introduction of contaminants which can remain in the solid phase and might be eluted in subsequent steps; and (c) the excess of confidence in the cleanup capacity of the procedures which lead to the absence of checking and control steps.

Liquid–solid extraction steps have been aimed at cleaning and concentration of the analytes prior to either individual separation by LC or selective quantitation by CPBA [50,60] or RIA [61–63]. The improvement in both selectivity of the solid phases and subsequent instrumentation (namely, HPLC–MS or GC–MS hybridisation) and selective reagents (namely monoclonal antibodies) has simplified dramatically the cleanup and preconcentration steps. A general overview of liquid–liquid extraction and liquid–solid extraction sample pretreatment (including type of immiscible phase and solid phase nature, respectively) is shown in Table 1. The basic designs for liquid–solid extractions have been grouped depending on the use of single [9,13,14,33,35,36] or dual cartridges [14,66]. The type of chromatography and the detection system used are also listed in Table 1. The contribution of the authors of this review to liquid–solid cleanup-preconcentration procedures has been the automation of methods for the sequential determination of 25-OH-D_3, 24,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 in serum by integrating on line in a continuous flow manifold the solid phase (aminopropyl-silica) cleanup-preconcentration

steps with the subsequent individual HPLC separation and UV detection [64–67]. The sample only requires an initial deproteinization step by liquid–liquid extraction with an isopropanol–hexane mixture followed by evaporation of the organic solvent and redissolution in an appropriate buffer. These automated procedures constitute a promising, inexpensive approach for the routine determination of the metabolites in clinical samples.

(E) Pretreatment based on liquid chromatography. This is a relatively frequent step prior to individual chromatographic separation, sometimes difficult to distinguish between them. Molecular exclusion [24,53], solid–liquid partitioning either by normal [13–15] or reverse phase chromatography [24,27,69] or a combination of some of them [27,53,69] have been used for the development of the former step, which has sometimes been followed by RIA (for the quantitation of 1,25-$(OH)_2$-D_3 [62]) and CPBA (for 24,24-$(OH)_2$-D_3 [33]. Another different method reported by Withold et al. for the determination of 1,25-$(OH)_2$-D_3 is based on immunoextraction of the analyte prior to quantitation by RIA [70].

4. Quantitation methods of vitamin D_3 and its metabolites

The evolution of the methods for the determination of vitamin D_3 and its metabolites has been the response to the necessity of a more in depth knowledge of their biological behaviour which has required more selective and sensitive ways for quantitation as a result. The basis of the evolution has been the spectacular development of the analytical instrumentation in the last decades. An overview of the type and frequency of the appearance of new methods in the last two decades is shown in Fig. 5, the most salient of which are commented below:

(A) Methods based on protein saturation. The principle of these methods is the case with which

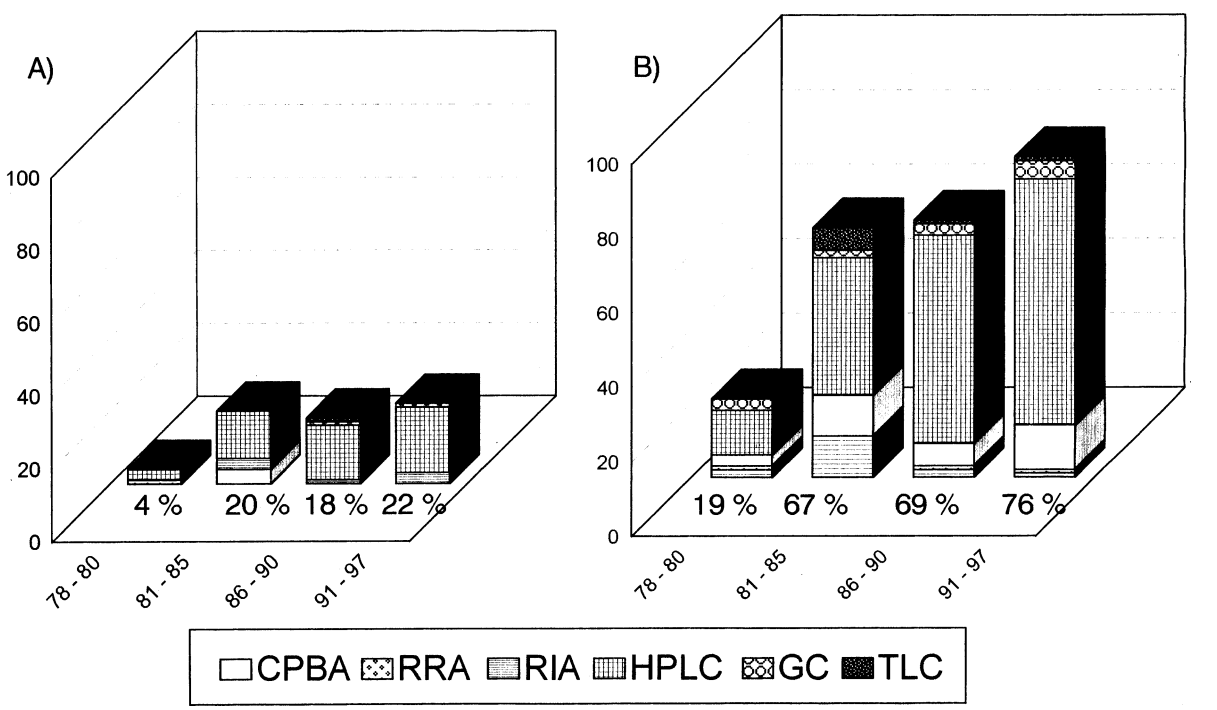

Fig. 5. Evolution of the determination methods on vitamin D_3 and its metabolites during the last two decades (for abbreviations, see text).

secosteroids are being bound to proteins yielding very stable complexes. The earliest protein saturation reactions gave place to very non-specific bonds, a fact that was circumvented by laborious liquid–liquid and liquid–solid separation steps followed by complicated multidimensional chromatography. The characterisation of specific proteins at the beginning of the 1980's resulted in their analytical application [71]. An abundant literature on direct application of competitive protein binding assays (CPBA) coupled to photometric detection [59,71–80], or radioreceptor assay (RRA) for the individual or overall determination of the vitamin D_3 hydroxymetabolites is at present available.

Antigen–antibody interactions, characterised by high selectivity (even specificity) and sensitivity are a useful tool for the determination of these metabolites at the low concentrations they exist in very complex matrices, such as serum and plasma. The routine use of this tool is restricted by the scarce proliferation of monoclonal antibodies specific for these metabolites. Most of the research in this respect has been devoted to 1,25-$(OH)_2$-D_3 determination [70,81,82]; on the contrary, most of the immunoassays proposed for the determination of 25-OH-D_3 are based on non-specific, heterogeneous immunoassays which require time-consuming pretreatment steps [31,83–85]. Radioimmunoassays based on the use of two antibodies (usually an antiserum one from goat and a more selective anti-goat antibody, which is obtained by a second host) and a tracer (an iodide derivative of a C_{22} carboxylic acid), have also been reported [81].

(B) Methods based on chromatographic separation. Liquid chromatography (LC) has been considered as the most useful technique for the separation of hydroxyvitamin D_3 metabolites. HPLC has been principally used either as an additional cleanup procedure previous to the application of a specific method for quantitation of each one of the hydroxymetabolites or as a definitive separation/detection approach using an appropriate on line detection system. The first application of HPLC for the determination of vitamin D_3 or its metabolites appeared in the 1970's as a multi-dimensional approach using normal and reversed-phase modes coupled with quantitation procedures based on saturation analysis, radioreceptors and UV detection. Several authors have developed cleanup/separation approaches based on the alternative use of LC separation and liquid–liquid extraction [29]. The development of new commercial solid-phase cartridges based on solid–liquid extraction has also contributed to the development of new HPLC methods based on the use of a single-column [86,87] or two sequential columns [35,37,57,73]. On the other hand, HPLC based on both polar and non-polar solid phases has also been used. In this way, normal-phase chromatography for the hydroxymetabolites of D_3 vitamin has been based on the use of silica sorbents [35,37,54,86,87] and hexane/isopropanol as mobile phase; reverse phase chromatography has been developed by a non-polar hydrocarbon sorbent such as C_8 [8,27,88] or C_{18} [8,9,45,86,89] and isocratic or gradient elution mixtures of methanol/water [8,45,86] or acetonitrile/water [27,88]. Recently, HPLC based on the use of calcium phosphate hydroxyapatite and lead phosphate hydroxyapatite as new solid-phases has been proposed for the determination of cholecalciferol and related substances [90,91].

The coupling of HPLC with very diverse detection systems has been proposed for the development of new methods for the determination of vitamin D_3 and its metabolites. UV detection based on single wavelength has been proposed for the direct monitoring of 25-OH-D_3 [7,24,35,37,87,89] or prior to its conversion into isotachysterol derivatives [93]. A dual-wavelength monitoring approach [86] has also been reported for the detection of vitamin D hydroxymetabolites. Recently, integrated systems based on HPLC with fluorescence detection and pre- or post-column derivatization using Cookson type reagents (PTAD or DMEQ-CON) for the determination of 25-OH-D_3 in serum have been reported [8,9,15,88]. Several LC methods based on electrochemical detection have been also proposed [45,94]. One of these for the determination of 1,25-$(OH)_2$ and 25-hydroxy-16-ene-23-yne vitamin D_3 was based on normal-phase HPLC using CH_2Cl_2/hexane/THF/isopropanol (35:10:5:3) as

mobile phase, merging with an electrolyte stream of tetrabuthylammonium hexafluorophosphate [94] followed by hydrodynamic voltammetric detection at a 3 mm vitreous-carbon working electrode which operated at 0.8–1.5 V versus Ag/AgCl. Another approach for the determination of vitamin D_3 and 25-OH-D_3, was based on coupling LC with amperometric detection at 1.05 V versus Ag/AgCl, using $LiClO_4$ as both mobile phase and electrolyte [45].

The coupling between LC separation and MS detection provided definitive methods for the quantitation of hydroxyvitamin D_3 compounds. These methods were mainly based on pre- or post-column derivatization using Diels-Alder cycloaddition reactions [10,27]. Yeung et al. [10] have proposed a method for the determination of vitamin D_3 and its hydroxymetabolites using liquid chromatography, continuous flow and fast atom bombarding in a tandem coupling with MS (i.e. HPLC–CF–FAB–MS–MS), while Brinkman et al. and Wilson et al. have based their methods for the same analytes on the PTAD ability for enrichment of the ionization signal by electrospray (ESI) and thermospray (TSP), respectively [27].

Finally, the scarce application of GC to the determination of vitamin D_3 [79], and its metabolites is remarkable [13,14,16,17,20,21,92,95]. All of the methods reported have been based on GC–MS hybridation that should be sufficiently sensitive for quantitation of the main metabolites of vitamin D_3. However, GC produces complicated chromatograms due to non-specific dehydration as a consequence of their isothermal cyclation occurring at the high temperatures reached in the column oven. This drawback has been circumvented by prior conversion into a more stable derivative due to dehydration or silanization reactions. Making et al. have developed different approaches in this sense. In 1988, the same authors developed the first method for the determination of vitamin D_2, D_3 and their metabolites based on trimethylsilanization and final formation of *n*-butylboronate derivatives prior to GC–MS [13,14]. Recently, these authors proposed a mass fragmentography–GC method for the determination of 25-hydroxyvitamin D_2 and D_3 in plasma [20]. After purification, capillary-gas chromatography mass-spectrometry was carried out following dehydration of the analytes on an aluminium powder pre-column at 400°C.

5. Present and future prospects

The relatively limited number of analytical contributions presented in the X Workshop on Vitamin D being celebrated last year in Strasbourg gives clear evidence of the problems with which the research groups are faced in the development of new more sensitive and selective methods for the determination of vitamin D and its metabolites [96–99]. The most remarkable aspects of the evolution of the methods for the determination of vitamin D_3 and its hydroxymetabolites are as follows:

1. Decreased complexity of the cleanup/preconcentration procedures and non-specific measurements based on protein saturation and scintillation detectors by changing to simple solid-phase separation procedures, on-line derivatization and more sensitive and selective detectors (fluorimetry and mass spectrometry, among others).
2. Multi- versus single determinations. The simultaneous determination of several vitamin D_3 related compounds affords a more solid establishment of an almost complete pattern of the status of vitamin D_3 in the organism.
3. More frequent use of liquid chromatography as both a cleanup and separation-quantitation tool.
4. New pre- and post-derivatization steps based on Diels-Alder cycloaddition.
5. Limited use of GC, which has been coupled to MS in most cases.
6. Disappearance of methods based on protein saturation and radioreceptors.
7. New, very selective immunoassays based on the use of monoclonal antibodies, particularly for 1,25-$(OH)_2$-D_3.

The methods to be developed in order to fulfil the necessities of hydroxyvitamin D_3 metabolite determination must be supported on the milestones of the present analytical chemistry: multidetermina-

tion, automation and miniaturisation [100]. The following aspects should be considered:

(1) Improvement and simplification of the cleanup and sample pretreatment steps. Simpler procedures for sample cleanup and preconcentration of analytes are mandatory prior to the development of semi- and automated procedures for cleanup/preconcentration. The improvements of sample cleanup and preconcentration based on the use of semi-automated flow injection approaches with HPLC separation and UV-detection [64–68,96] constitute an open way in which a deeper research should be developed.

(2) Development of new equipment based on the instrumental hyphenation between separation techniques and highly selective detectors and the synthesis of new reagents for use in fluorimetric [97] or MS [97,98]. Derivatization must be hence improved.

(3) New immunoassays based on the synthesis of highly selective monoclonal antibodies especially for 1,25-$(OH)_2$-D_3 [99]. The scarce development of monoclonal antibodies against other hydroxyvitamin metabolites such as 25-OH-D_3 calls for attention of the researchers in this field.

(4) Development of external quality assurance programs, in order to establish interlaboratory comparisons thus improving the quality of the determination of the hydroxymetabolites, mainly in clinical applications. The development of the EQAS program [99], with more than 30 participants, is a first step in this sense.

(5) Multideterminations, is one of the most important trends in this area. The multidetermination of the vitamin D_3 hydroxymetabolites would provide a global knowledge of the vitamin D status and so a better diagnostic. On the other hand, the development of new structural analogues of these metabolites has been presented as one of the most acute trends in clinical and therapeutic applications of vitamin D making mandatory the development of new methods for the simultaneous determination.

(6) The development of new procedures for data acquisition and treatment, must be presented as one of the best ways of increasing sensitivity in the determination of vitamin D metabolites. The use of procedures for high discrimination and amplification via the incorporation of computing and mathematical routines would probably improve the sensitivity of the methods for the determination of these metabolites present in human fluids at very low concentrations.

(7) Finally, the use of new analytical techniques, may contribute to an increase of both sensitivity and selectivity. Particular interest would be paid to the use of supercritical fluid extraction [101] as advantageous alternative for sample cleanup/preconcentration in clinical applications. On the other hand the use of high energy lasers as excitation sources for UV or fluorimetric detection should lead to an increase of the sensitivity in the vitamin D hydroxymetabolites determination, taking advantage of laser induced fluorescence [102–104].

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