Determination of Dissociation Constants of Some Hydroxylated Benzoic and Cinnamic Acids in Water from Mobility and Spectroscopic Data Obtained by CE-DAD

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In this study, the dissociation constants of a series of phenolic acids, including benzoic acid, four hydroxylated derivatives (*p*-hydroxybenzoic, protocatechuic, vanillic, and gallic), and four hydroxylated cinnamic acid derivatives (*p*-coumaric, caffeic, ferulic, and sinapinic) have been determined in water by capillary electrophoresis (CE) from the electrophoretic mobilities at different pH value. The pK_a values have also been obtained from the absorbance spectra at the maximum of the electrophoretic peaks, which were measured with a diode-array detector (CE-DAD methodology). The pK_a values obtained from both methodologies (CE and CE-DAD) have been compared with those previously published in the literature and also with the values predicted by the SPARC online pK_a calculator.

Introduction

Phenolic and polyphenolic compounds are a complex group of natural substances that are present in plants, fruits, and vegetables[1] and in derived foods and contribute to their organoleptic properties.[2] There is a growing interest in the characteristics and behavior of these substances because of their beneficial health effects, such as their antimutagenic, antioxidant, or antimicrobial properties[3−6] or their presumed role in the prevention of various degenerative diseases, such as cancer and cardiovascular diseases.[7] In fact, the protection role of vegetables and fruits is attributed to their contents in bioactive antioxidant compounds that act as scavengers against free radicals,[8,9] which are thought to be responsible for many age-related diseases.[9]

For these reasons, a variety of analytical methods dealing with the determination of these compounds in foods, vegetables, and plants, based mainly on liquid chromatography and electrophoretic separations, are described in the literature.[2,7,10−13]

Many biologically active molecules are fully or partially ionized at physiological pH, and it has often been shown that the presence of charged groups is necessary for biological activity, solubility, or both. Therefore, the ionization constant, pK_a, is a very important physicochemical parameter of a substance; knowing this parameter is of fundamental importance in a wide range of applications and research areas, particularly in analytical chemistry, where a satisfactory knowledge of the acid−base behavior of substances is essential for predicting the influence of pH on liquid chromatography and capillary electrophoresis separations.[14−19]

Capillary electrophoresis (CE) has been introduced as a convenient method for the separation of different substances. In the case of ionizable compounds, their electrophoretic behavior is established from the relationships between the electrophoretic mobility of each species, the dissociation constants of the substances, and the pH of the buffer solution. This allows the acidity constants of the substances by measuring the electrophoretic mobility as a function of pH.[20,21] The absorbance detection in CE also makes possible the pK_a determination from absorbance/pH data obtained at a single wavelength.[22−24] Another procedure for the determination of dissociation constants in CE is based on the use of a diode array detector (CE-DAD); in this case, the absorbance data are measured at the maximum of the electrophoretic peaks as a function of the pH of the electrophoretic buffer.[25,26] In this way, the dissociation constants can be determined from either electrophoretic mobilities as a function of pH (CE) or the absorbance spectra as a function of pH (CE-DAD).

In this work, both approaches have been used for the determination of the dissociation constants of a series of nine phenolic acids. They include benzoic acid, four hydroxylated derivatives (*p*-hydroxybenzoic, protocatechuic, vanillic, and gallic acids), and four hydroxylated cinnamic acid derivatives (*p*-coumaric, caffeic, ferulic, and sinapinic). The structures of the compounds studied are given in Scheme 1.

The pK_a values obtained have been compared with those previously obtained from potentiometric and spectrophotometric data[27] and with the predicted values by the SPARC online calculator, which estimates several physicochemical properties of organic compounds from their molecular structure.[28,29]

Experimental Section

Chemicals. The phenolic acids were supplied by Sigma-Aldrich (Madrid, Spain) and were used without further purification. Electrophoretic buffers covering the pH range of 4.0 to 11.0 at 0.5 pH unit intervals were prepared from analytical reagent grade commercial products: CH_3COOH (pH 4 to 5), H_3PO_4 (pH 5.5), tris(hydroxymethyl) aminomethane (TRIS) (pH 7 to 8.5), H_3BO_3 (pH 9 to 10), and NaOH from Merck (Darmstadt, Germany) and 2-(*N*-morpholino) ethane sulfonic acid (MES) (pH 6) and 3-(cyclohexylamino) propanesulphonic

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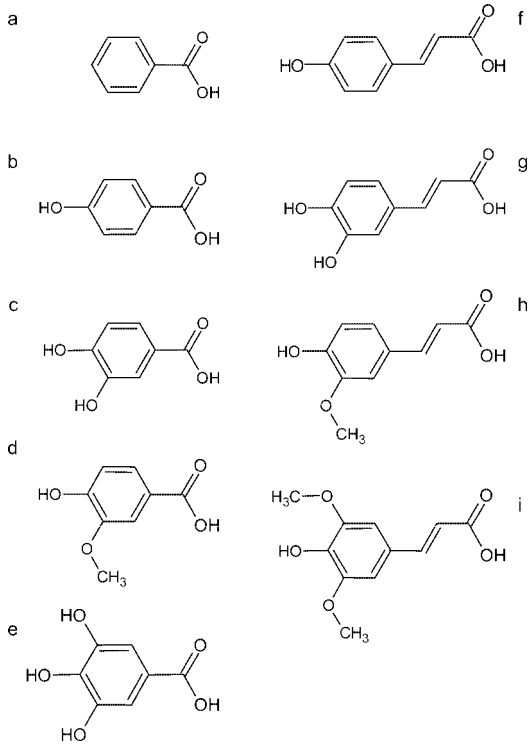
Scheme 1. Structures of the Acids Studied[a]



[a] a, benzoic; b, *p*-hydroxybenzoic; c, protocatechuic; d, vanillic; e, gallic; f, *p*-coumaric; g, caffeic; h, ferulic; i, sinapinic.

acid (CAPS) (pH 10.5 to 11) from Sigma-Aldrich (Madrid, Spain). All buffer concentrations were 0.025 M, except in the case of the borate buffer (0.05 M).

Stock standard solutions of phenolic acids in water (about 200 $\mu g \cdot mL^{-1}$) were diluted to obtain the working solutions at concentrations of 50 $\mu g \cdot mL^{-1}$. Acetone (3 % v/v, Merck) was added to every phenolic acid solution as an electroosmotic flow (EOF) marker.

All solutions were prepared with deionized water (Milli-Q deionizer, Millipore, Bedford, MA).

Apparatus. Electrophoretic experiments were performed on a P/ACE system 5500 (Beckman Instruments, Palo Alto, CA) equipped with an autosampler, an automatic injector, and a photodiode array detector. Beckman System Gold Software was used to record the experimental data (electropherograms and absorption spectra).

A (47 cm)·(75 μm) i.d. untreated fused-silica capillary, (Polymicro Technologies, Phoenix, AZ) was used. The temperature of the capillary was kept at 298.2 K by a liquid coolant in the capillary cartridge. The experiments were performed at 20 kV.

The pH of the buffer solutions was measured with a CRISON 2002 potentiometer (Crison Instruments, Barcelona, Spain) using a Ross 81-02 combination electrode (Orion Research, Boston, MA). The electrode system was calibrated with pH 4.008 and 6.863 buffer solutions according to DIN 19266.

Procedure. The capillary was activated by the pressure injection of 1 M sodium hydroxide solution for 30 min, followed by a 30 min rinse with ultrapure Mill-Q water and 30 min with the running electrolyte. When the buffer was changed, the capillary was successively rinsed for 5 min with water, 20 min with 1 M NaOH solution, 20 min with water, and 30 min with the new buffer solution. The last step was the application of a 20 kV voltage for 15 min with the capillary filled with buffer solution. To equilibrate the capillary and thereby minimize hysteresis effects, the capillary was flushed between runs with ultrapure water for 1 min and then with running buffer for 3 min. Furthermore, at the beginning of each day, the capillary was purged with 1 M NaOH solution for 5 min, followed by water for 5 min and working buffer solution for 20 min.

For each pH assayed, working solutions of phenolic acids were hydrodynamically injected at 0.5 psi for 4 s in triplicate for several days to obtain constant electrophoretic mobilities. The criterion applied was that the differences in electrophoretic mobilities on 3 consecutive days should be less than $2 \cdot 10^{-6}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$.

Electrophoretic mobilities (m_e) were determined from the migration time of the neutral marker (t_{eof}), the migration time of each phenolic acid (t), the length of the capillary (L_C), the length of the capillary between the injection end and the detector (L_D), and the applied voltage (V) according to the following equation

$$m_e = \left(\frac{L_C L_D}{V}\right)\left(\frac{1}{t} - \frac{1}{t_{eof}}\right) \quad (1)$$

The absorption spectra at the maxima of the electrophoretic peaks were recorded between (200 and 350) nm at 3 nm intervals.

Data Treatment. With the exception of benzoic acid, the compounds studied can be considered to be diprotic acids in the pH range studied. Therefore, their effective electrophoretic mobility can be obtained from the electrophoretic mobilities of each possible species and their corresponding mole fractions

$$m_e = m_{e,H_2A}\chi_{H_2A} + m_{e,HA^-}\chi_{HA^-} + m_{e,A^{2-}}\chi_{A^{2-}} \quad (2)$$

These values can be related to the stoichiometric dissociation constants (K'_a) and the hydrogen ion concentration of the buffers according to the following expressions

$$K'_{a,1} = \frac{[HA^-][H^+]}{[H_2A]} \quad (3)$$

$$K'_{a,2} = \frac{[A^{2-}][H^+]}{[HA^-]} \quad (4)$$

$$m_e = m_{e,H_2A}\frac{[H^+]^2}{[H^+]^2 + K'_{a,1}[H^+] + K'_{a,1}K'_{a,2}} + m_{e,HA^-}\frac{K'_{a,1}[H^+]}{[H^+]^2 + K'_{a,1}[H^+] + K'_{a,1}K'_{a,2}} + m_{e,A^{2-}}\frac{K'_{a,1}K'_{a,2}}{[H^+]^2 + K'_{a,1}[H^+] + K'_{a,1}K'_{a,2}} \quad (5)$$

$$m_e = \frac{m_{e,H_2A}[H^+]^2 + m_{e,HA^-}K'_{a,1}[H^+] + m_{e,A^{2-}}K'_{a,1}K'_{a,2}}{[H^+]^2 + K'_{a,1}[H^+] + K'_{a,1}K'_{a,2}} \quad (6)$$

Moreover, because that the neutral species (H_2A) has zero electrophoretic mobility, eq 6 can be simplified to

$$m_e = \frac{m_{e,HA^-}K'_{a,1}[H^+] + m_{e,A^{2-}}K'_{a,1}K'_{a,2}}{[H^+]^2 + K'_{a,1}[H^+] + K'_{a,1}K'_{a,2}} \quad (7)$$

However, the dissociation constants described in eqs 3 and 4 are conditional constants, defined as concentration quotients. In our case, the ionic strength of buffers is not constant; therefore, there are small changes in the values of the stoichiometric dissociation constants for the different buffers.

This can be overcome by using thermodynamic dissociation constants (K_a), defined as activities quotients. They are related to the stoichiometric dissociation constants and the activity coefficients (γ_i) by

$$K_{a,1} = \frac{a_{HA^-} a_{H^+}}{a_{H_2A}} = \frac{\gamma_{HA^-}[HA^-]\gamma_{H^+}[H^+]}{\gamma_{H_2A}[H_2A]} = K'_{a,1}\frac{\gamma_{HA^-}\gamma_{H^+}}{\gamma_{H_2A}} \quad (8)$$

$$K_{a,2} = \frac{a_{A^{2-}} a_{H^+}}{a_{HA^-}} = \frac{\gamma_{A^{2-}}[A^{2-}]\gamma_{H^+}[H^+]}{\gamma_{HA^-}[HA^-]} = K'_{a,2}\frac{\gamma_{A^{2-}}\gamma_{H^+}}{\gamma_{HA^-}} \quad (9)$$

For diluted solutions, the activity coefficients can be obtained by the Debye−Hückel equation

$$\log \gamma_i = -\frac{Az_i^2\sqrt{I}}{1 + a_0 B\sqrt{I}} \quad (10)$$

where z_i is the charge of the i species, I is the ionic strength of the buffer, the A and B parameters are a function of temperature and dielectric constant of the medium,[30] and a_0 is the radius of the solvated ion. For aqueous solutions at 298.2 K, $A = 0.51$, and the product a_0B is set to 1.5. The activity coefficient of the uncharged species (H_2A) is assumed to be equal to unity.

The pK_a values from m_e/pH data could be determined from the inflection points of the m_e/pH curves, but the pH intervals used here (0.5 units) do not allow a precise estimation. In this work, they have been determined by using the NLREG 4.0[31] software. This is a general nonlinear least-squares regression program in which a set of initial parameters is iteratively refined until a minimum of an objective function is attained. In our case, these parameters correspond to the thermodynamic pK_a values and the mobilities of the ionic species (m_{e,HA^-} and $m_{e,A^{2-}}$); the objective function, U, is defined as the sum of the squared differences between the experimental and predicted values of the effective mobilities ($m_{e,exptl}$ and $m_{e,pred}$, respectively) obtained in each electrophoretic buffer

$$U = \sum_{i=1}^{n} (m_{e,exptl,i} - m_{e,pred,i})^2 \quad (11)$$

where n indicates the number of data pairs m_e/pH obtained as the mean of three replicates. The predicted mobilities ($m_{e,pred}$) are calculated from the dissociation constants and individual electrophoretic mobilities, the measured pH of buffers, and the calculated activity coefficients from the ionic strength of buffers. Initial estimates for p$K_{a,1}$ and p$K_{a,2}$ can be obtained from the inflection points of the m_e/pH curves, and the initial values for $m_{e,HA^-}$and $m_{e,A^{2-}}$ were taken from the experimental m_e/pH data (at intermediate and high pH values, respectively).

Moreover, an independent set of dissociation constants was determined from the absorption spectra recorded at the maxima of the electrophoretic peaks; in this case, absorbance data depend on the pK_a values, the pH of buffers, and the molar absorptivities of the different species. The experimental data were processed with the program STAR.[32] This is a nonlinear regression program that was specifically developed for the study of complex equilibria from spectrometric data. It allows the refinement of equilibrium constants from data containing as many as 150 spectra measured at up to 50 wavelengths. This program uses the Gauss−Newton algorithm, and it refines the equilibrium constants until a minimum value in the sum of the squared differences between the experimental ($A_{i,j,exptl}$) and calculated ($A_{i,j,calcd}$) absorbances is reached

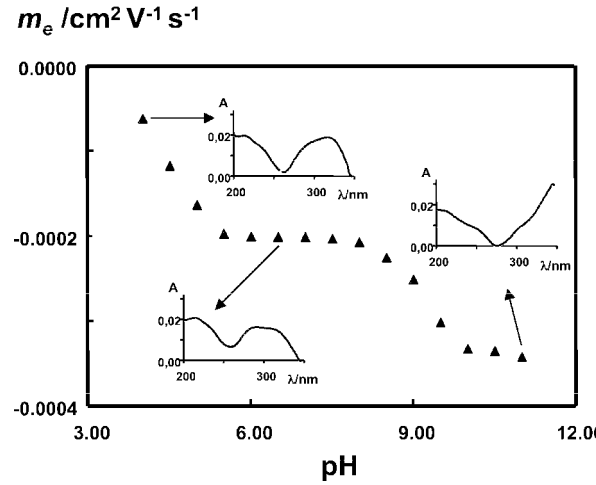


Figure 1. Plot of electrophoretic mobilities (▲) of sinapinic acid against pH. The absorption spectra were recorded at pH values: 4.0, 6.5, and 11.

$$U = \sum_{i=1}^{ns}\sum_{j=1}^{nw}(A_{i,j,exptl} - A_{i,j,calcd})^2 = \sum_{i=1}^{ns}\sum_{j=1}^{nw}\left(A_{i,j,exptl} - \left(\sum_{k=1}^{p}\varepsilon_{k,j,calcd} b C_{k,i,calcd}\right)\right)^2 \quad (12)$$

where ns and nw indicate the number of solutions studied (number of spectra) and wavelengths, respectively. The number of species p is equal to 3 in this case (H_2A, HA^-, and A^{2-}), and b is the path length. The calculated absorbances are obtained in several steps.

First, the program solves the mass balances for each spectrum according to the initial estimates of equilibrium constants and the experimental conditions; this gives a set of calculated concentrations for each species C. ($C_{k,i,calcd}$ indicates the computed concentration of the species, k, in the solution, i.)

The second step consists of the determination of the unknown molar absorptivities (ε matrix) from the computed species distribution and the experimental absorbances; this is done following a multiple linear regression procedure. The calculated absorbance values are determined from the matrices C and ε, and they are used to determine the objective function, U (eq 12).

Now, the Gauss−Newton refinement procedure is applied by using a numerical differentiation to obtain a new set of equilibrium constants, which define a lower U value. The process is repeated until a minimum of U is reached. The results consist of a set of refined equilibrium constants and a set of molar absorbances for each species, which satisfy the minimum in the sum of squared errors.

Results and Discussion

As noted before, the absorption spectra are recorded at the maximum of the electrophoretic peaks (procedure CE-DAD). Therefore, the spectra obtained at different pH can also be used for the pK_a determination, provided that the different species (H_2A, HA^-, and A^{2-}) have different absorption spectra.

Figure 1 is an example of the experimental data recorded for sinapinic acid, showing the experimental electrophoretic mobilities as a function of the pH of buffers, together with the recorded spectra at three selected pH values.

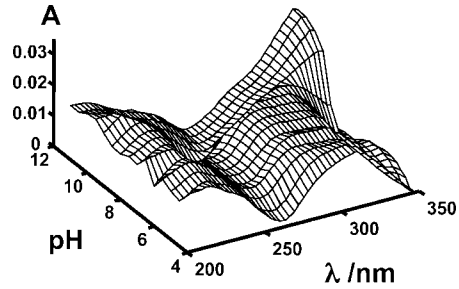


Figure 2. Absorption spectra obtained at the maxima of electrophoretic peaks of sinapinic acid at different pH buffers.

The absorption spectra for the same substance, obtained in the pH range of 4.0 to 11.0, are presented in Figure 2.

The full set of experimental data m_e/pH for all compounds studied (CE data) is plotted in Figure 3, together with the expected electrophoretic mobilities from the pK_a values obtained.

The results obtained from CE and CE-DAD data are given in Table 1, which contains the computed electrophoretic mobilities for the ionic species and the dissociation constants. The Table also gives the pK_a values reported in the literature, together with those predicted by the program SPARC.

The comparison of these values indicates, in general, a good agreement with the data corresponding to the first dissociation constant, with maximum differences of about 0.2 pK_a units between CE, CE-DAD, SPARC values, and literature data. The exception is the value obtained for protocatechuic acid, which gives a higher value (about 0.3 pK_a units) when calculated from CE-DAD than when obtained from CE data.

However, there is a higher dispersion for pK_{a2} values: CE and CE-DAD results are close, in general, with the exception of the results corresponding to vanillic, caffeic, and sinapinic acids, in which the pK_a values obtained from CE-DAD are about 0.3 units higher than those obtained from CE data. This effect does not seem to be a trend because the pK_{a2} of sinapinic acid obtained from CE-DAD (9.20) is equivalent to the result obtained from potentiometric data (9.21) and close to the value determined from spectrometric data (9.40). However, caffeic acid shows very different values for pK_{a2} depending on the procedure used for pK_a determination: 8.51 (CE), 8.83 (CE-DAD), or 8.32 (potentiometric). The SPARC predicted values for pK_{a2} are less reliable than those obtained for pK_{a1}. In fact, only the predicted pK_{a2} values for *p*-coumaric and ferulic acids are close to experimental data, and those for protocatechuic, gallic, and sinapinic acids are very low (between (0.8 and 1.2) pK_a units lower than experimental values).

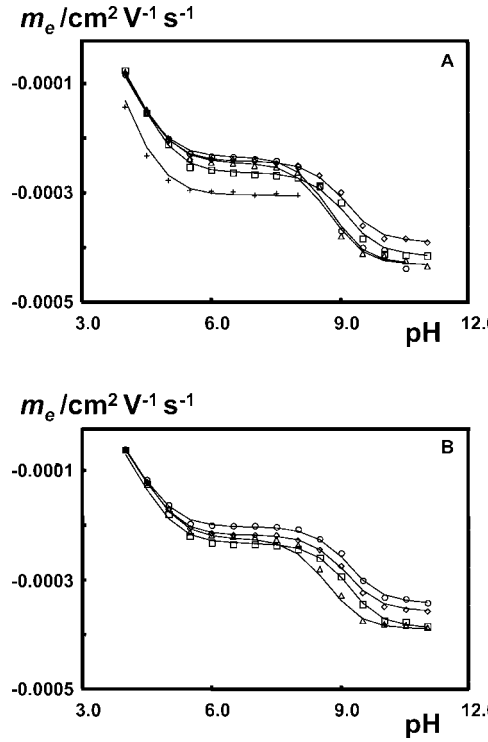


Figure 3. Experimental electrophoretic mobilities of the substances studied as a function of pH of buffers. (A) Benzoic acid derivatives: +, benzoic; □, *p*-hydroxybenzoic; △, protocatechuic; ◇, vanillic; ○, gallic. (B) Cinnamic acid derivatives: □, *p*-coumaric; △, caffeic; ◇, ferulic; ○, sinapinic. Solid lines are the calculated electrophoretic mobilities after the dissociation constants given in Table 1 (CE results).

The results obtained in this work indicate that the CE-DAD methodology is a useful procedure in the determination of dissociation constants from CE: it allows two kinds of data sets (m_e/pH) and (*A*/pH) to be obtained, and these can be used for independent pK_a determination. Therefore, a comparison can be made between two procedures to explore and understand the obtained results; this is important because both data sets are obtained in the same experimental run.

Moreover, from the comparison of the results obtained from the diprotic acids studied in this work, it can be concluded that the first dissociation constant is a parameter that can be readily obtained from either the literature or the use of the SPARC program to predict the value. However, in the case of accurate pK_{a2} requirements, SPARC results are less reliable, and a comparison of literature data is needed to check the experimental procedure that is most similar to the required experimental conditions.

Table 1. Electrophoretic Mobilities (m_e) and Dissociation Constants of Phenolic Acids at 298.2 K[a]

	CE results				CE-DAD results		potentiometric		spectrometric		SPARC prediction	
compd	m_{e,HA^-}[b] 10^{-4} cm²·V⁻¹·s⁻¹	$m_{e,A^{2-}}$[b]	p$K_{a,1}$[b]	p$K_{a,2}$[b]	p$K_{a,1}$[b]	p$K_{a,2}$[b]	p$K_{a,1}$	p$K_{a,2}$	p$K_{a,1}$	p$K_{a,2}$	p$K_{a,1}$[c]	p$K_{a,2}$[c]
benzoic acid	−3.03(2)		3.92(2)		4.15(9)		4.20[d]		4.19[f]		4.06	
p-hydroxybenzoic acid	−2.63(4)	−4.25(6)	4.26(4)	8.84(8)	4.25(7)	9.10(2)	4.38[e]	8.97[e]			4.30	8.68
protocatechuic acid	−2.45(4)	−4.36(6)	4.22(5)	8.61(9)	4.54(10)	8.53(5)	4.38[e]	8.74[e]	4.35[g]	8.79[g]	4.19	7.86
vanillic acid	−2.42(3)	−3.97(5)	4.17(4)	8.81(8)	4.04(10)	9.08(2)	4.31[e]	8.81[e]	4.16[h]	8.96[h]	4.08	8.54
gallic acid	−2.32(6)	−4.36(10)	4.11(8)	8.47(11)	4.22(10)	8.62(6)	4.24[e]	8.67[e]			4.09	7.30
p-coumaric acid	−2.34(3)	−3.89(4)	4.34(3)	8.83(6)	4.55(15)	8.84(10)	4.39[e]	8.37[e]	4.36[h]	8.98[h]	4.32	8.97
caffeic acid	−2.18(5)	−3.94(6)	4.30(6)	8.51(10)	4.48(8)	8.83(5)	4.47[e]	8.32[e]			4.30	8.14
ferulic acid	−2.17(2)	−3.63(3)	4.30(3)	8.81(3)	4.38(8)	8.75(4)	4.56[e]	8.65[e]			4.27	8.83
sinapinic acid	−2.01(3)	−3.47(4)	4.25(4)	8.89(6)	4.27(8)	9.20(4)	4.40[e]	9.21[e]	4.19[h]	9.40[h]	4.21	8.04

[a] The estimated standard deviations are given between parentheses in last digit units. [b] This work. [c] Ref 28. [d] Ref 33. [e] Ref 34. [f] Ref 35. [g] Ref 36. [h] Ref 27.

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