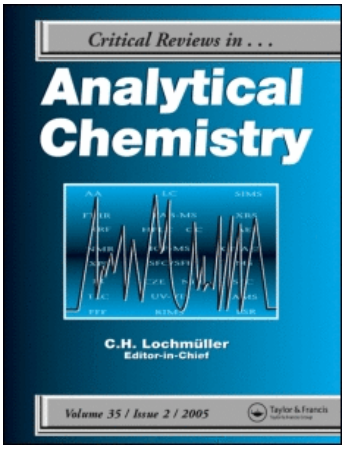

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Analytical Procedures for Determination of Quercetin and its Glycosides in Plant Material

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Quercetin and its glycosides are widely distributed in the plant kingdom and belong to the most abundant of the flavonoid molecules. Besides their important biological roles in plant pigmentation, these flavonols possess anti-cancer and anti-inflammatory properties, which are the consequence of their affinity for proteins and their anti-oxidant properties. The content of quercetin and its glycosides in different plants have been summarized in various papers, although quantitative data are not so reliable due to the wide diversity of extraction and determination procedures. The general analytical strategy involves the isolation from a sample matrix followed by separation, identification and measurement. The recovery step usually involves solvent extraction using a range of solvents. Separation is commonly achieved by high-performance liquid chromatography (HPLC) and capillary electrophoresis. This paper intends to present and discuss the analytical procedures for determination of quercetin—aglycone as well as its conjugates—in different plant materials by means of routine and more recently developed separation techniques. Selected applications are included to illustrate the scope and limitations of the various approaches.

INTRODUCTION

Flavonoids, particularly quercetin and its derivatives, have received special attention as dietary constituents during the last few years. The epidemiological studies point out to their possible role in preventing cardiovascular diseases and cancer (1–3). Flavonoids behave as anti-oxidants by a variety of ways including direct trapping of reactive oxygen species, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in processes forming radicals and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals (1, 4). The flavonoids of dietary significance are widely distributed in the plant kingdom and can be categorized as flavonols, flavanols, flavanones, flavones, anthocyanidins and isoflavones.

Quercetin, 3,3′,4′,5,7-pentahydroxylflavone, presented in Fig. 1, is one of the most abundant flavonoids present in fruits and vegetables. In plants, it occurs mainly in leaves and in the outher parts of the plants as aglycones and glycosides, in which one or more sugar groups is bound to phenolic groups by glycosidic bonds. Glucose is the most common sugar, with galactose and rhamnose also frequently found in composition with flavonoids. In general, quercetin glycosides contain a sugar group at the 3-position. Glycosylation increases the polarity of the flavonoid molecule, which is necessary for storage in plant cell vacuoles (5). A considerable amount of isoquercetrin (quercetin-3-*O*-β-glucoside) has been found in apple and pear peels (6) as well as in *Hypericum perfloratum* leaves or flowers (7). Almost 180 different glycosides of quercetin have been described in nature, with rutin (quercetin-3-*O*-β-rutinoside) being one of the most common (8, 9). In onion, one of the common quercetin sources, however, the phenolic group at the 4′-position is necessarily bound by a sugar group and thus its major glycosides are quercetin 4′-O-β-glucoside and quercetin 3,4′-O-β-diglucoside (Fig. 1). Their content is about 80% of the total content of flavonoids. Vegetables, fruits and red wine are the main dietary sources of quercetin. Onion (*Allium cepa* L.) ranked highest in quercetin content in a survey of 28 vegetables and nine fruits (10). In fruits and vegetables, flavonols and their glycosides are found predominantly in the skin where they serve, among others, as ultra-violet protection.

Nevertheless, we should take into account the fact that food-derived quercetin could be presented in its glysocides form and thus the effectiveness of its anti-oxidant activity is greatly modified by the position of the sugar group attached to the basic diphenylpropane structure. Quercetin aglycone seems to be a more active chain-breaking anti-oxidant than, its glycoside counterparts because of its higher accessibility to the site of chain-initiating and chain-propagating free radicals in

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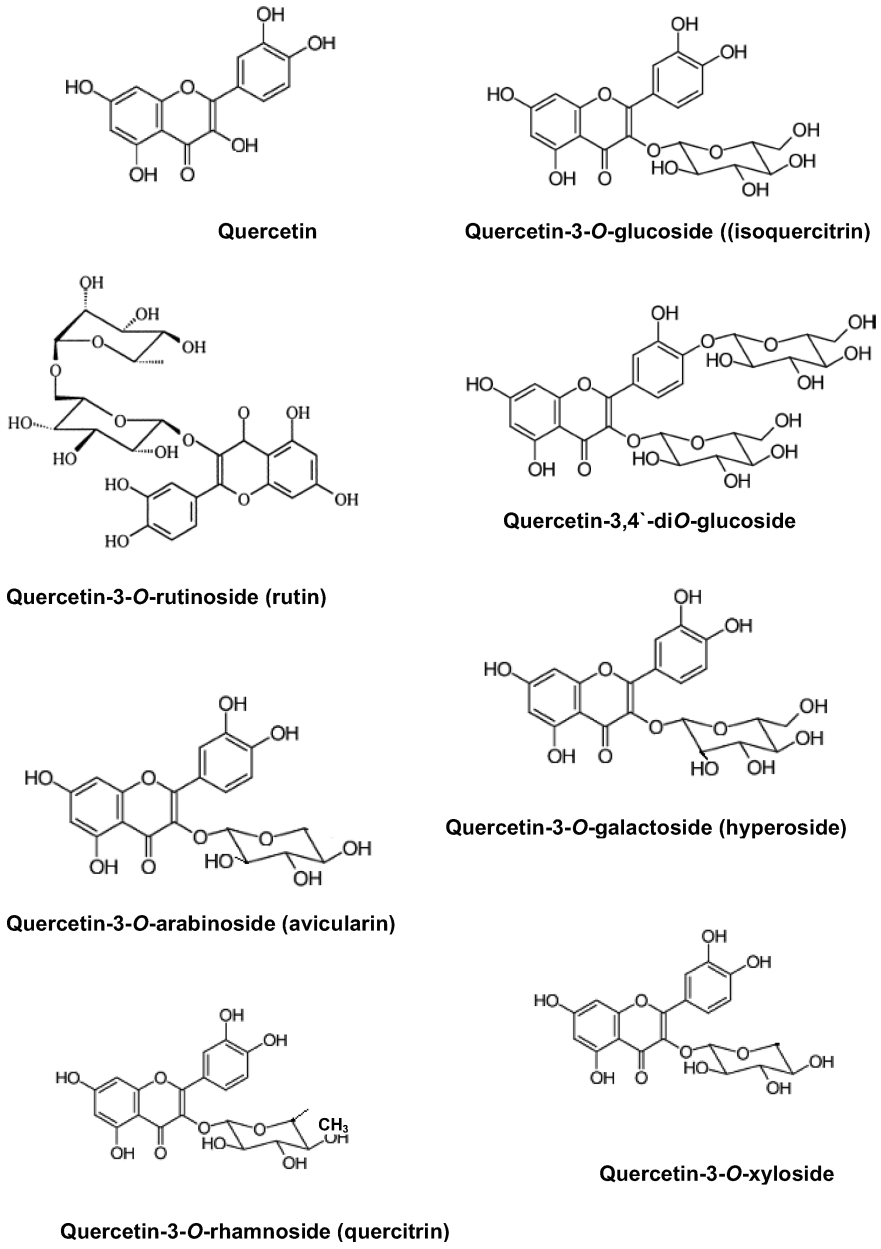

FIG. 1. The structures of quercetin and its major glycosides.

membranous phospholipid bilayers (4). It should be mentioned that total anti-oxidant capacity in the phenolic mixture is almost equal to the sum of anti-oxidant capacity of its individual phenolic compounds (11).

The content of quercetin and its glycosides in different plants have been summarized in various papers (8, 12–14), although quantitative data are not so reliable due to the wide diversity of extraction and determination procedures. The general analytical strategy involves the isolation from a sample matrix followed by separation, identification and measurement. The recovery step usually involves solvent extraction using a range of solvents. Separation is commonly achieved by high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), although gas chromatography is used in some instances. The most common mode of separation exploits reversed-phase (RP) systems typically with a C_{18} column and various mobile phases. Detection is routinely achieved by ultraviolet absorption often involving a photodiode detector and various mass spectra methods.

The present paper intends to present and discuss the determination of quercetin—aglycone as well as its conjugates—in different plant materials by means of routine and more recently

developed analytical techniques. In all instances, selected applications will be included to illustrate the scope and limitations of the various approaches.

SAMPLE COLLECTION, STORAGE AND PREPARATION

Due to the vast reservoir of plants, and the variation of their different parts, it is impossible to have any definitive procedure or protocol for the collection and storage of all plant materials. However, sample collection and storage conditions are essential and should be treated carefully, because plant variety, as well as growing location and season, may significantly affect the quantity and quality of our analysis. Most plants used in traditional medicine are harvested and dried for storage, while at other times, fresh or frozen plant material have been used. The processing and storage conditions, such as drying temperature and duration, storage length and humidity, therefore may affect the outcome.

Flavonoids are generally found at higher concentrations in outer layers of fruits and vegetables, therefore peeling results in their great loss. After home-like peeling, red onions contained 79% of the original total content of quercetin-4′-glucoside, while this compound was unaffected by chopping of onions (15). DuPont et al. (16) found that shredding of lettuce leaves followed to exposure to light produced significant losses of quercetin glycosides ranging 6–94% depending on the lettuce variety.

Storage experiments with commercial cultivars of onion were performed at a low constant temperature (1°C) and at higher variable temperatures (similar to 8°C) (17). Cultivar differences in quercetin glucoside content were significant but neither nitrogen fertilizer level nor lifting time had more than minor effects at start of storage or after 3 or 5 months of storage. The role of onion size was inconsistent but seemed to be of minor importance.

The changes in the content of some major flavonoids, such as quercetin and kaempferol, in onions, green beans and peas before and after different heat treatment (blanching, water-cooking, cooking in a microwave oven, frying and warm-holding of the water-boiled sample at 60°C for 1–2 hours) were studied by Ewald et al. (18). The significant losses of quercetin took place during the peeling and trimming process of onions (39%). The onions were of different size and they were peeled very unequally which means that several layers were peeled off from some onions and only one layer from others. Consequently, the losses of flavonoids were very high in the onions where several layers were peeled off since the most of quercetin is present in the first and second layer of this vegetable. The small changes of flavonoid content at further processing of all onion samples were within the observed biological variation among samples.

The pH value had an important influence on the degradation process of quercetin during heating in aqueous solution (19). At pH 5 the amount of quercetin decreased to approximately 75% after 300 minutes and under weak basic conditions (pH 8) was undetectable by HPLC/DAD after 240 minutes (Fig. 2). The

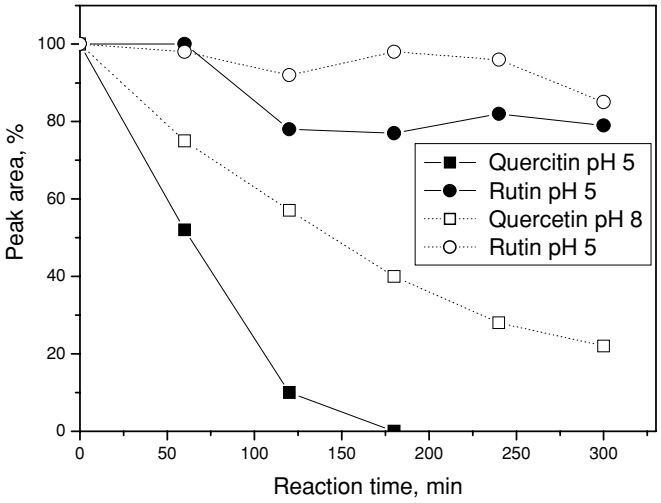

FIG. 2. Degradation of quercetin and rutin (1 mM) in aqueous solution at 100°C at different pH (19). (Copyright John Wiley & Sons Limited. Reproduced with permission)

presence of oxygen accelerates the degradation process. Rutin, the quercetin glycoside, showed a higher stability towards oxidation (Fig. 2). Lombard et al. (20) reported that baking and sauteing of onion produced a 7–25% gain in quercetin concentration, while boiling produced an 18% decrease in the content of this flavonol.

Rhodes and Price (21) investigated the compositional changes of flavonols in onions (skinned, chopped and immediately frozen in liquid nitrogen) during autolysis at 25°C over the 24-hours period. Their results showed that there was only a small loss (11–18%) in total 4-*O*-flavonoids content. However, in all examined varieties of onion there were significant quantitative changes in flavonoid composition. For brown-skinned onion, for example, within 2 hours 19% of quercetin-3,4-diglucose was lost with the appearance of an equimolar amount of quercetin-4-glucose, whilst during the first 5 hours there was a 50% decrease of the diglucosides accompanied by increases in both the monoglucoside and the free aglycone. All of the diglucosides had disappeared by the end of 24 hours.

Carrots retained more flavonoids after 30 days storage when, before drying, were treated with the solution containing ascorbic acid (0.1%) and glucose (1%). (22). Over this period about 10% of flavonoids content was lost when carrots were then dried in a hot-air oven at 80°C in comparison to freeze-drying at –50°C.

EXTRACTION

Simple filtration for some liquid samples, such as fruit juices and wines, is ineffective for recovering quercetin and its glycosides and alternative strategies are necessary. The advantage of liquid extraction in comparison with direct injection have been demonstrated for HPLC analysis of a wine sample (23). Quercetin and its glycosides from fresh, freeze-dried and air-dried samples are generally extracted with pure methanol,

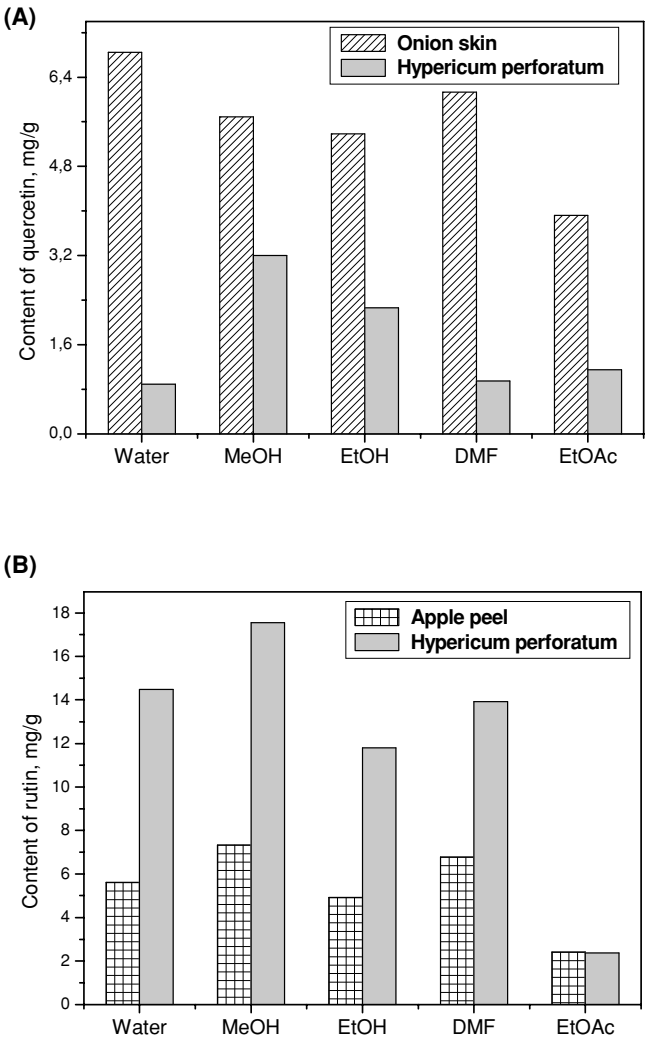

FIG. 3. The efficiency of extraction of (A) quercetin and (B) rutin using different solvents (26). (Copyright Elsevier Limited. Reproduced with permission).

ethanol or their combination with water (10, 24–27), but in some cases ethyl acetate (28) or acetone (6, 29) have been used.

The efficiencies of different solvents for extraction of quercetin and rutin from onion skin, apple peel and *Hypericum perforatum* leaves (expressed in mg per gram of the dry plant material) are presented in Fig. 3 (26). The extract composition was the same in each case, but the flavonoids yields were different. The highest efficiency from onion samples was obtained when water or DMF, the most polar solvents, were used. At the sub-cellular level, phenolic compounds are located mainly in the vacuoles. Their occurrence in soluble or suspended forms, and in combination with cell wall components, may have a significant impact on their extraction (30). With dried plant materials, such as *Hypericum perforatum*, low polarity solvents, such as ethyl acetate, simply leach the sample whereas alcoholic solvents or water presumably rupture cell membranes and enhance the extraction of endocellular materials (25). Aqueous methanol as an extracting solvent proved to be a useful compromise that ensures the extraction of both aglycones and flavonoid glycosides, depending on time and temperature (24). Aqueous methanol, due to its polarity, is more effective for extraction of polyphenols linked to polar fibrous matrices, while acetone-water mixtures are more useful for extracting polyphenols from protein matrices, since they appear to degradate the polyphenol-protein complexes (29).

Extraction of tea polyphenols was usually done by steeping tea leaves in boiling water for 5–30 minutes (31–33). The infusion was then filtered and subjected to HPLC analysis. On the other hand, tea polyphenol extracts were obtained using pure methanol (34) or by employing a multiple extraction of tea samples with 80% (v/v) methanol and subsequently with 80% methanol containing 0.15% HCl (35).

The possibility of quercetin degradation under different solvent and temperature conditions was tested by Pinelo et al. (36). An initial increase and a following decrease in its anti-radical activity was observed in ethanol and methanol solutions when storage time was prolonged. By contrast, a progressive decrease in anti-oxidant activity was determined in 10% (v/v) ethanol water solution due to oxidative cleavage which is favored under these conditions.

The alternative extraction methods such as sonication (37–39), supercritical fluid extraction (40, 41) and pressurized liquid extraction (42), due to shorter extraction time and reduced solvent consumption, have gained increasingly popularity for isolation of quercetin and its glycosides. A review on the ultrasonically assisted extraction of active compounds from plant materials was published (43). Due to apolar properties of CO_2 in supercritical fluid extraction the significant amount of polar organic modifiers has to be added to obtain high extraction yield; however, it leads to reduced selectivity (44, 45). For example, in extracting polyphenols from green tea, the best extraction yield was found in the system using 95% ethanol and 5% CO_2 (44). Comparison of the effectiveness of Soxlet extraction, sonication and pressurized-fluid extraction of several major phenolic compounds in St. John's wort herb is presented in Fig. 4 (39).

Many extracts contain significant portions of carbohydrates, lipids and other unwanted compounds that potentially interfere with subsequent quantification. Simultaneous sample clean-up and pre-concentration of the analytes could be achieved by solid-phase extraction (SPE) using a range of sorbents (13, 24, 25, 46). In most cases, C_{18}-bonded silica or polymeric sorbents are used and the sample solution and solvents are usually slightly acidified to prevent ionization of the flavonoids, which would reduce their retention. Methanolic extract of the homogenized olives was evaporated to dryness, redissolved in water containing hydrochloric acid (pH 2) and loaded on a C_{18} sorbent (47). After washing with hexane to remove lipids, the flavonoids were eluted with pure methanol. C_{18} cartridges were also used for isolation and pre-concentration of quercetin from the extracts of vegetables (48) and cranberry juice (49). The use of

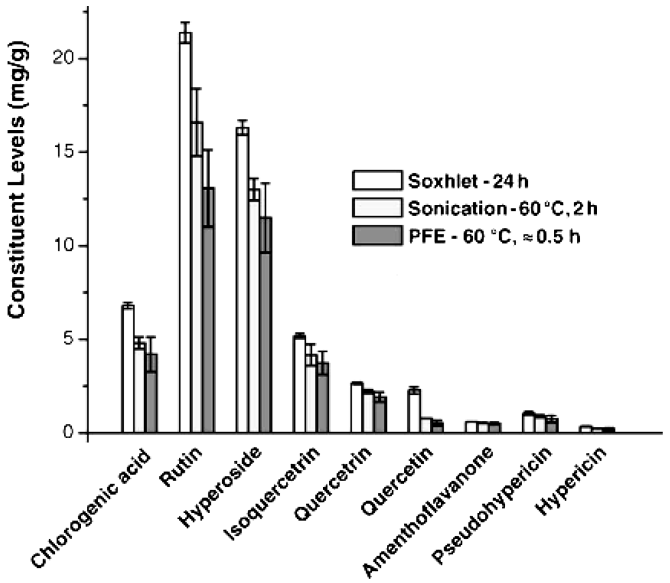

FIG. 4. Comparison of the effectiveness of Soxlet extraction, sonication and pressurized-fluid extraction (PFE) of several major phenolic compounds in St. John's wort herb (39). (Copyright Elsevier Limited. Reproduced with permission).

polymeric sorbent Oasis HLB (Waters, Milford, MA, USA) effectively eliminated the interfering constituents from biological samples with efficient extraction of quercetin (50). Amberlite XAD-2 (Aldrich, Deisenhofen, Germany) particles (51, 52) and Bond Elut C_{18} (Varian, Middelburg, The Netherlands) cartridges (53) have been utilized for purification of acidified solutions of honey. Molecularly imprinted polymers (MIPs) are novel sorbents used for SPE of phenolic compounds in food samples (54–56). Analyte is used as a template during formation of the polymer following appropriate extraction, which leaves receptors for a molecular recognition of targed analyte. Figure 5

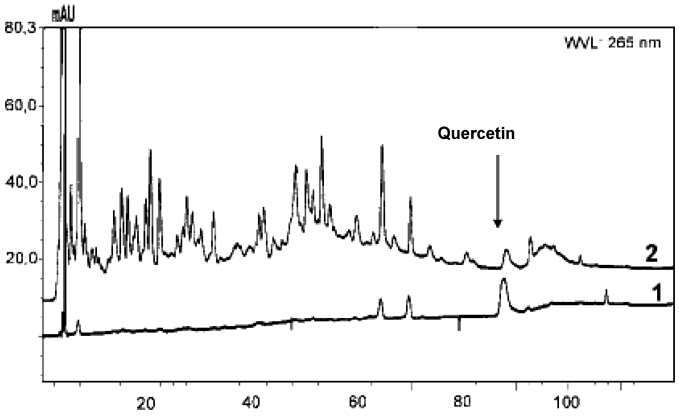

FIG. 5. Chromatographic analysis of Merlot wine (1) with and (2) without molecular imprinted polymer. Elution was done with acetonitrile (54). (Copyright American Chemical Society. Reproduced with permission).

shows that the use of MIPs greatly reduced the complexity of chromatographic analysis of red wine and enhanced the intensity of quercetin peak (54). Recently, similar MIPs for rutin isolation from wine, tea and juice samples has been presented (56).

Matrix solid-phase dispersion (MSPD) has been also tested for the analysis of naturally occurring constituents from different plant materials providing, in many cases, equivalent or superior results to more classical solvent and SPE techniques (57, 58). The entire sample is homogeneously dispersed into particles of very small size, usually a C_{18} or C_8-bonded silica, providing an enhanced surface for subsequent extraction of the components. However, this technique does not give sufficient results for all phenolic compounds. Xio et al. (59) compared the efficiency of MSPD with ultrasonic and soxlet extraction for isolation of isoflavonoids from Radix astragali, the dried root of a medicinal Chinese plant. While the amount of the aglycones were higher by MSPD, the extraction efficiency of the glycosides were better using the conventional techniques, particularly solvent extraction. Extraction of quercetion-3-*O*-glucoside from white grapes with MSPD in just one step gave similar results in comparison to combined isolation procedure with solvent extraction (aqueous HCl, pH 2, containing 5% methanol, 40°C, in an ultrasonic bath) followed by a clean-up step with C_{18} non-endcapped sorbent and ethanol elution (60). The best recoveries of aglycone quercetin in all the assays (different sample to sorbent ratios) carried out by MSPD were around only 30%.

HYDROLYSIS

Often due to difficulties in later separation procedures, hydrolysis of quercetin glycosides before or during extraction is performed to break the glysocidic bonds and to obtain the aglycone form. The extraction recovery is dependent on molarity of HCl, hydrolysis time and temperature and the composition of the extraction solvent. In most publications the hydrolysis of flavonoid glycosides from vegetables and fruits is carried out in 1.2 M HCl at 90°C for 2 hours according to a procedure presented by Hertog et al. (10). However, the extended exposure time to HCl could cause degradation of quercetin (10, 61). Generally, for the hydrolysis process, the optimum compromise is to achieve complete release of aglycones and to minimize degradation reactions of compounds involved. For this purpose, a central composite experimental design was described (62). Applying a multiple regression analysis on the data set, it was possible to obtain a mathematical model that took linear, quadratic and cross-product terms into account. According to this mathematical approach, optimum conditions for rutin hydrolysis in orange juice corresponded to HCl concentration of 1.5 M and a hydrolysis time of 1 hour.

Identical optimum hydrolysis conditions for flavonol glucosides in different vegetables or fruits could not be achieved as they are dependent on the binding site of the sugar on the flavonoid nucleus (10). The study presented by Lombard et al. (20) indicated that the main quercetin glucosides present in onion were relatively heat stable, thus, higher temperature and shorter time for their hydrolysis could be employed. However,

it should be mentioned that the conditions resulting in optimal breakdown of glycosides could be too harsh for some of the other phenolic compounds present in the same plant material. For example, myricetin as well as catechins could be degradated at high temperatures (20, 63). The method of choice is always a compromise between efficiency production of aglycone from the plant material and degradation of aglycones.

Nuutila et al. (63) compared the efficiency of hydrolysis of quercetin glycosides from red onion and spinach in the presence of *tert*-butylhydroquinone (TBQH) and ascorbic acid as the anti-oxidants. Although TBQH gave a slightly higher concentration of quercetin than ascorbic acid, it caused some interferences with the detection. Moreover, the higher addition of ascorbic acid than 2 mg acted as a pro-oxidant rather than as an anti-oxidant. Thus, the proposed method (2 hours refluxing at 80°C with 1.2 M HCl in the presence of ascorbic acid) could be applied for screening vegetables and leafy vegetables but would probably not be suitable for samples, which have higher concentrations of myricetin and phenolic acids.

SEPARATION

In general, separations of quercetin and its glycosides have been mainly carried out by HPLC equipped with RP columns, generally packed with spherical particles of silica bonded with octadecyl (C_{18}) chains (13, 24, 25, 46). HPLC columns packed with monolithic supports, consisting of a single piece of porous material, are alternative means of performing fast separations. The main advantage of this type of support is its excellent hydrodynamic property, which allows the reduction of backpressure and increases the flow rate. Monolithic columns are increasingly being applied in phytochemical analysis (64). However, in the field of food analysis they were only used for determination of phenolics compounds in wine (65) and phenolic acids in fruits (66). A highly hydrophilic poly(7-oxonorbornene-5,6-dicarboxylic acid-*block*-norbornene)-coated silica was also investigated for the liquid chromatographic determination of flavonoids in plant extracts (67). Compared to the most commonly used octadecyl-derivatized silica this sorbent allowed fast separation even at extreme pH values.

Gradient elution is usually used in recognition of the complexity of the phenolic profile of plant samples (68–76). Numerous mobile phases have been employed but binary systems comprising an aqueous component and a less polar organic solvent, such as acetonitrile or methanol, remain common. Acid, such as acetic, formic or trifluoroacetic (TFA), is usually added to maintain constant acid concentration during gradient runs (Table 1). Phosphate buffers are less popular, mainly because of the dreaded contamination of ion sources when mass spectrometry detection is used. In some cases, isocratic elution has provided adequate resolution due to selectivity effects of one or more components of the mobile phase (77). The elution pattern for flavonoids is flavanone glycoside followed by flavonol and flavone glycosides and then the free aglycones in the same order. Most of these methods have been developed to measure different groups of polyphenolics in a single plant material or a few groups in multiple plant sources, but obtaining good resolution is considered to be the main difficulty in this last approach. The selected examples of application of HPLC to the determination of quercetin and its glycosides in plant materials are presented in Table 1.

Ultra performance liquid chromatography (UPLC) takes advantage of technological strides made in particle chemistry performance (78). Using 1.5–2 μm particles, a more narrow analytical column and instrumentation that operates at higher pressures than those used in HPLC, drastic increases in resolution, sensitivity and speed of analysis can be obtained. The same separation on RP-HPLC that takes over 20 minutes can be accomplished in under 3 minutes by UPLC. This new chromatographic methodology has been applied in plant material analysis so far for separation and quantification of the major chocolate polyphenols (79).

Although HPLC stays as the most dominating separation technique for polyphenolic compounds, CE is gaining popularity and represents an alternative method for the analysis of plant materials (12, 24). The CE modes primarily used for these purposes are capillary zone electrophoresis (CZE) (80–88) and micellar electrokinetic chromatography (MECK) (89, 90).To achieve ionization of hydroxy compounds (as relatively weak acids) enabling their separation by CZE, background electrolytes based on borate buffer of pH 9–11 are widely used and in many cases simultaneously the complex formation ability of B(III) is utilized for manipulating or enhancing the selectivity of electrophoretic separation. Molybdenate was also examined as a complex-forming additive (91). It forms more stable complexes with aromatic o-dihydroxy compounds and hence the complex-formation effect is observed at a considerably lower pH equal to 5.4. CZE is only applicable to charged analytes and the charge-to-size ratios determine the electrophoretic migration times. In MECK for neutral analytes in the presence of surfactants such as sodium dodecyl sulfate, separation is based on hydrophobicity, which affects the analyte partitioning between the aqueous phase (moving with the electro-osmotic flow) and the micellar phases (charged and migrating with a different velocity). For ionic analytes separation in MECK is based on both the degree of ionization and the hydrophobicity. Wang et al. (92) assessed and compared the electrophoretic behavior of 13 flavonoids commonly found in medicinal plants using these two modes of CE. The separation selectivity of MECK was shown to be better than that of CZE, because electrophoretic behavior in the latter is affected by more factors, such as the degree of saturation and the stereochemistry of the C-ring, alkyl substitution and the number and position of phenolic hydroxy groups, methylation and glycosylation of the hydroxy groups, as well as the complexation of flavonoids with a borate buffer. Unfortunately, the authors did not study any natural samples. Non-aqueous capillary electrophoretic separation of a group of flavonoids was investigated in methanol at high. pH to alter selectivity of the separation (93). Additionally, tests of untreated capillaries and capillaries coated

TABLE 1
Selected examples of application of HPLC to the determination of quercetin and its glycosides in plant materials

Sample	Detected compounds	Stationary phase	Mobile phase	Detection	Ref.
Onion, spinach	quercetin, Q-3-rut, Q-3-glu	Symmetry C18, 5 μm (150 × 3.9 mm)	Gradient: 2–60% (v/v) methanol with aqueous TFA (300 μL/L)	DAD	63
Strawberries	quercetin	Purospher RP-18e, 5 μm (125 × 3 mm ID)	Gradient: A-1% formic acid, B-acetonitrile	DAD	68
Onion, apple, blubbery,	quercetin, Q-3-rut, Q-3-rha	LiChromosorb RP18, 7 μm (25 × 4 mm)	Gradient: A-0.05% aqueous TFA, B-0.05% TFA in acetonitrile	DAD	69
Herbs	quercetin, Q-3-rut, Q-3-gal, Q-3-glu, Q-3-rha	YMC ODS-AQ RP-18, 5 μm (250 × 4.6 mm)	Gradient: A-0.5% TFA + 20% (v/v) methanol, B-acetonitrile	DAD	70
Artichoke	quercetin, Q-3-rut, Q-3-gal, Q-3-rha, Q-3-glu	Luna C18, 3.5 μm (50 × 2.1 mm)	Gradient: A- 0.1% formic acid, B-acetonitrile with 0.1% formic acid	DAD, MS	71
Apple pomace	Q-3-rut, Q-gal, Q-3-glu, Q-3-xyl, Q-3-ara*p*, Q-3-ara*f*, Q-3-rha	Aqua C18, 5 μm (25 × 4.6 mm)	Gradient: A-2% (v/v) acetic acid, B-0.5% acetic acid in water and acetonitrile (50:50, v/v)	MS, NMR	72
Mango	Q-gal, Q-3-glu, Q-3-ara, Q-3-rha	LiChromospher RP 18e, 5 μm (250 × 4 mm)	Gradient: 2% (v/v) acetic acid in water B-0.5% acetic acid and acetonitrile (50:50, v/v)	DAD, MS	73
Cashew apple	Q-3-gal, Q-3-glu, Q-3-xyl*p*, Q-3-ara*p*, Q-3-ara*f*, Q-3-rha	Waters C18, 5 μm (250 × 4.6 mm)	Gradient: A- 0.05% acetic acid, B-acetonitrile	DAD, MS	74
Apple, pear	Quercetin, Q-3-rut, Q-3-gal, Q-3-glu, Q-3-xyl, Q-3-ara, Q-3-rha	Aqua C18, 5 μm (250 × 4.6 mm)	Gradient: A- 2% acetic acid, B-acetonitrile	MS	75
Onion	Q-4′-glu, Q-3,4′-diglu, Q-7,4′-diglu, Q-3-glu, Q-3,7,4′-triglu	Discovery C18, 5 μm (250 × 4.6 mm)	Gradient: acetonitrile in water	DAD	76

Q-3-gal—quercetin-3-laactoside (hyperoside); Q-3-glu—quercetin-3-glucoside (isoquercetrin); Q-3-ara—quercetin-3-arabinoside (avicularin); Q-3-rha—quercetin-3-rhamnoside (quercetrin); Q-3-xyloside; Q-3-rut—quercetin-3-rutinoside [rhamnosyl(α 1→6)-glucoside] (rutin); Q-3-xyl*p*—quercetin-3-xylopyranoside; Q-3-ara*p*—quertcetin-3-arabinopyranoside (gujaverin); Q-3-ara*f*—quercetin-3-arabinofuranoside.

TABLE 2
Application of capillary electrophoresis to the determination of quercetin and its glycosides

Sample	Detected compounds	Background electrolyte	Detection	Ref.
Medicinal plants	Q-3,4′-diglu, Q-4′-glu	25 mM sodium tetraborate with 20% (v/v) methanol, pH 9.3	UV (270 nm)	80
Onion	Q-3-rut, Q-3-rha	10 mM boric acid, 10 mM sodium tetraborate, 15 mM EDTA in 15% (v/v) methanol, pH 10.2	UV (280 nm)	81
Red wine	Quercetin, Q-3-rut, Q-3-rha	25 mM β-hydroxy-4-morpholinoprpanesulfonic acid, 50 mM Tris, 15 mM boric acid and 5 mM β-cyclodextrin, pH 8.5	UV (254 nm)	82
Mulberry leaves	Quercetin, Q-3-rut, Q-3-rha	150 mM boric acid, pH 10	UV (270 nm)	83
Medicinal plant	Quercetin, Q-3-rut	50 mM sodium tetraborate in 18% (v/v) methanol, pH 9.7	UV (210 nm)	84
Propolis	Quercetin, Q-3-rut	30 mM sodium tetraborate, pH 9	UV (214 nm)	85
Standard mixture	Quercetin, Q-3-rut, Q-3-glu	0.1 M boric acid – NaOH, pH 10	UV (254 nm)	86
Medicinal plants	Quercetin	30 mM sodium tetraborate in 8% (v/v) acetonitrile, pH 9	UV (210 nm)	87
Grapes	Quercetin	35 mM sodium tetraborate, pH 8.9	UV (250 nm)	88
Wines	Quercetin	40 mM sodium tetraborate, 40 mM sodium dodecyl sulfate, pH 9	UV (214 nm)	89
Tea	Q-3-glu	50 mM NaH_2PO_4, 50 mM sodium tetraborate, 20 mM sodium dodecyl sulfate in 10% (v/v) acetonitrile, pH 6	UV (278 nm)	90

Abbreviations as in Table 1

with poly(glycidylmethacrylate-co-*N*-vinylpyrrolidine showed the analysis to be faster (6.5 minutes vs. 25 minutes) and the repeatability better in the coated capillaries.

CE as a separation technique is still evolving and a new mode of separation, called capillary electrochromatography (CEC), has been developed. This hybrid method combined CZE and μ-HPLC (94). It bridges the advantages of both these techniques, offering a unique separation mode and exploiting a combination of chromatographic retention and electrophoretic mobility. Application of CEC was also explored for analysis of quercetin (95) and biologically relevant herbal flavonols (96) (Table 2).

DETECTION

Routine detection in HPLC and CE is typically based on measurement of UV absorption, often using photodiode array detection (DAD). A match of both ultraviolet-visible (UV-VIS) spectrum and retention time can lead to highly positive identification of the separated analytes. DAD detector is capable for simultaneously detection of chromatograms at different wavelengths. This feature significantly enhances the performance of the separation system, particularly when different groups of polyphenols are mixed in one sample. When proper wavelengths are chosen, e.g., at the maximum absorptions, all groups can be detected with the highest sensitivity. An appropriate selection of the detection wavelength can also allow the quantification of an unresolved or poorly resolved peak (46). However, the use of conventional approaches based on spectra is often limited when samples contain very similar compounds.

Detection based on fluorescence is generally more sensitive than UV absorption. Quercetin and its glycosides do not show native fluorescence, although Rodriguez-Delago et al. (23) reported optimum excitation (260 and 264 nm) and emission wavelengths (426 and 420 nm) for quercitrin and quercetin, respectively, but they could be used only for high concentration of these analytes. Many flavonoids, such as quercetin, can form fluorescent chelates with several cations such as Mg(II), Be(II), Zn(II), Sc(III), Ga(III), In(III) and Al(III), which could be used as the post-column derivatization reagents for HPLC with fluorescence detection (97–100). The limit of detection for determination of quercetin, based on the formation of its fluorescent complex with Al(III) (97) and Ga(III) (98), was found to be 0.15 and 16.2 μg/L, respectively. The study shows that the 3-hydroxyl-4-keto oxygen site is essential for fluorescence as rutin—containing a sugar bound to the 3-hydroxyl group—does not form a fluorescent chelate (97).

HPLC or CE with electrochemical detection can be a useful completion technique for determination of quercetin and its

glycosides because they are, as most flavonoids, electroactive at modest oxidation potentials (62, 101–104). Romani et al. (101) compared HPLC procedures with DAD and electrochemical detection (differential pulse voltammetry and amperometric biosensor with bare graphite screen-electrodes) for analysis of phenolic compounds in natural extracts derived from grape, olives and green tea. The most accurate data were obtained from HPLC-DAD analysis, while differential pulse voltammetry was considered a good and quick method for screening. Recently, multi-walled carbon nanotubes have been found to be a excellent electrode material for the determination of quercetin and rutin at trace levels due to their strong surface adsorption (105, 106).

For complete structural identification of the analytes, other detection techniques such as mass spectrometry (MS) with different ionization modes is often necessary (13, 24, 25, 46, 74, 107, 108). Soft electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) provides the molecular masses of chromatographically separated molecules and tandem MS-MS provides extra structure details of thermally labile, non-volatile polar phenolic compounds. Usually APCI is used more extensively with small molecule compounds (MW < 1200), while ESI works better with more polar and higher molecular weight compounds. It was experimentally confirmed that quercetin sensitivity with the ESI mode is better than that with the APCI mode (109). The possible reasons for this are that APCI can cause high chemical background noise for low mass compounds and thermal degradation can occur with labile compounds, such as quercetin, which would decrease the APCI sensitivity.

Bonaccorsi et al. (76) demonstrated that the on-line HPLC-DAD electron spray mass spectrometry (ESI-MS-MS) technique constitutes an accurate and easy methodology for the qualitative and quantitative analysis of quercetin glycosides in southern Italian red onions, quercetin-4′glucoside and quercetin-3-4′-diglucoside being the most abundant components. The presence of quercetin-3-7-4′-triglucoside was identified and confirmed by UV spectra and product ion scan experiments (Fig. 6). This compound exhibited an ESI-MS spectrum in the positive ion mode with four significant peaks, one centred at m/z 789 $[M + H]^+$, the second at m/z 627 $[M + H—162]^+$, the third at m/z 465 $[M + H—162—162]^+$ and the last one at m/z 303 $[M + H—162—162—162]^+$ (Fig. 6A). Four analogous peaks were produced in the negative ESI-MS analysis (Fig. 6B). The loss of three glycosil units shown in the ESI-MS spectra and the fragmentation pattern in the negative ESI-MS-MS analysis on the ion at m/z 301 (Fig. 6C) were indicative of a quercetin triglucoside. The hypsochromic shift observed in the UV spectrum with respect to aglycone quercetin value (252→265 nm) excluded substitution onto the 5-position of the A-ring and confirmed this conclusion. The presented methodology allowed them to obtain a characteristic profile of several quercetin glycosides also in other vegetables that are rich in these compounds.

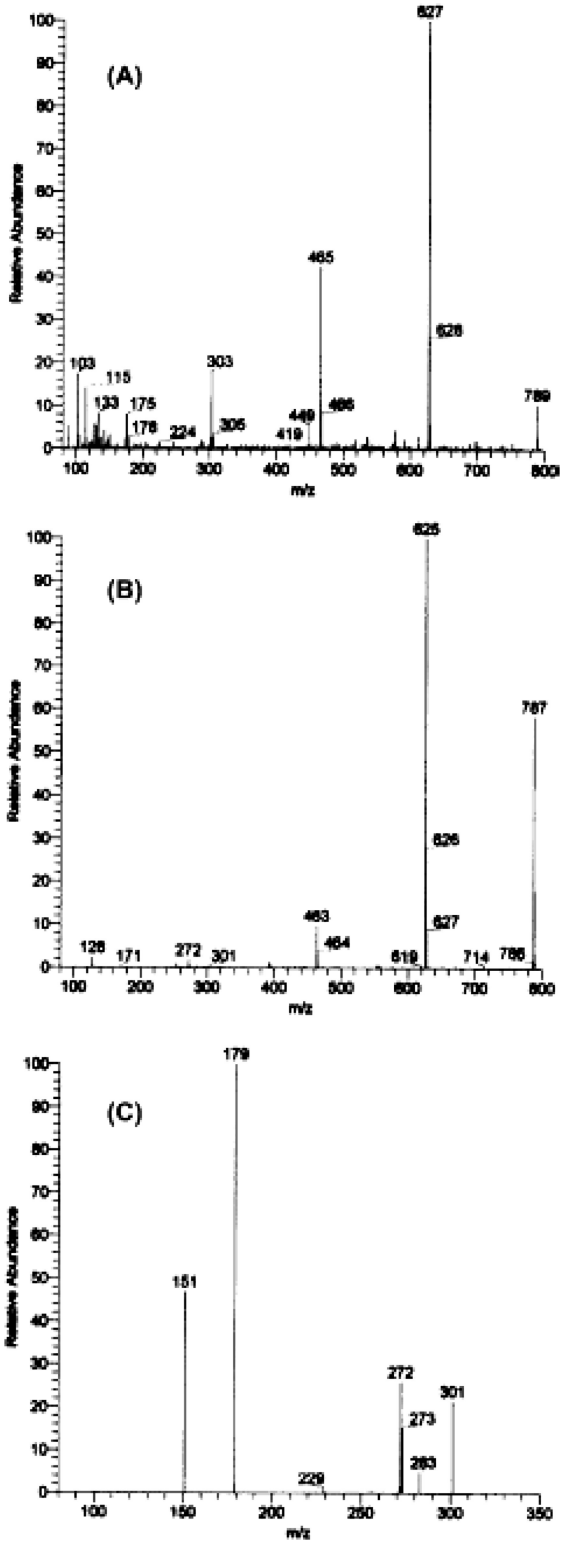

FIG. 6. ESI-MS spectra in (A) positive and (B) negative mode and (C) negative ESI-MS-MS spectrum of chromatographic peak corresponding to quercetin-3,7,4′-triglucoside (76). (Copyright American Chemical Society. Reproduced with permission).

CONCLUSION

Flavonoids are the main factors in the hypothesis that the consumption of a diet rich in vegetables and fruits may be protective for human health. The anti-oxidative properties of these compounds demonstrated in vitro become a key issue. Epidemiological studies have also shown an inverse effect between the intake of flavonoids and the risk of coronary heart disease. Quercetin belongs to the flavonol subgroup of flavonoids and is widely distributed as the main bioactive plant component. In plant food, quercetin exists in a multiplicity of complex conjugates, mainly glycosides. Important questions remain on the fate of such conjugates during processing and digestion, on the nature of the nutritionally important form of quercetin and on the extent of their uptake and metabolism in the gut (4, 9, 110). An important prerequisite for tacking such problems is the development of convenient and reliable analytical procedures with a high-resolution separation system and appropriate quantification.

The preliminary steps, such as isolation and extraction from plant materials, are of primary importance influencing the reliability and reproducibility of the analysis. For this purpose, several classical techniques, like solvent extraction, as well recent techniques (pressurized-fluid extraction and matrix solid-phase dispersion) have been tested. The important aspect of quercetin analysis is whether to determine the target analyte in its various conjugated forms or as the aglycone. Chromatographic or electrophoretic separation of the obtained extract, without hydrolysis is expected to result in the identification and quantification of the quercetin aglycone and its glycosides, simultaneously, in the presence of each other. Separation of the target analyte from a hydrolyzed extract provides a decreased number of compounds to be determined and the free aglycone form of quercetin, as well as quercetin, initially in glycosidic linkages cannot be distinguished. The combination of HPLC-DAD-MS or CE-DAD-MS is the best choice for separation, quantification and identification of the analytes. In order to define fine structural differences, homo- and heteronuclear NMR spectroscopy is also very helpful (111).

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