(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2012 (27.09.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/126789 A1

(51) **International Patent Classification**:
C07K 14/415 (2006.01) ***A01H 5/00*** (2006.01)
C12N 15/82 (2006.01)

(21) **International Application Number**:
PCT/EP2012/054506

(22) **International Filing Date**:
15 March 2012 (15.03.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
11158766.3 18 March 2011 (18.03.2011) EP

(71) **Applicant** *(for all designated States except US)*: **NESTEC S.A.** [CH/CH]; Avenue Nestlé 55, CH-1800 Vevey (CH).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **McCARTHY, James** [US/FR]; 298 Vallée de Vautruchot, F-37210 Noizay (FR). **LALLEMAND, Laura** [FR/FR]; 95, Rue Gabriel Péri, F-92120 Montrouge (FR). **McCARTHY, Andrew** [GB/FR]; 20, Rue Paviot, F-38120 Saint Egreve (FR).

(74) **Agent**: **LOMHOLT, Stig**; Avenue Nestlé 55, CH-1800 Vevey (CH).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/126789 A1

(54) **Title**: PRODUCTION OF CHLOROGENIC AND DACTYLIFRIC ACID SPECIES

(57) **Abstract**: Compositions and methods for making chlorogenic and dactylifric acid species, as well activated CoA thioester precursors are disclosed. Also disclosedare recombinant and mutant plant HCT, HQT, and 4CL enzymes for making such compounds in vitro or in vivo. Food products, such as coffee products,comprising exogenous chlorogenic and dactylifric acid species are also disclosed.

PRODUCTION OF CHLOROGENIC AND DACTYLIFRIC ACID SPECIES

BACKGROUND OF THE INVENTION

Field of the Invention

[001] The invention relates generally to phenylpropanoids, compositions and methods for producing chlorogenic and/or dactylifric acid species, and in particular to compositions and methods using plant HCT, HQT, and 4CL genes and proteins for making mono-, di-, and tri- and tetraesters and other complex chlorogenic and/or dactylifric acid species.

Description of Related Art

[002] Secondary metabolites in plants are not directly involved in primary cellular functions such as growth, photosynthesis or reproduction, but they are known to mediate plant-environment interactions and to play a major role in the plant's survival and adaptation to environmental changes. Three major classes of secondary metabolites, terpenoids, alkaloids, and phenolics, have been identified in plants.

[003] Structurally, the phenolics generally consist of a benzenoid ring bearing at least one hydroxyl substituent. As with all secondary metabolites the carbon skeletons can be modified by substrate-specific and/ or regio-specific biosynthetic enzymes (e.g. hydroxylases, methyltransferases, glycosylases etc.), resulting in a high structural diversity. More than 6,000 different phenolics have been identified. They serve a variety of important functions in plants, for example, pigmentation for the attraction of insect pollinators (anthocyanins), protection against solar radiation damage (hydroxycinnamic acids and flavones), defence against pathogens (stilbenes), signalling (flavonoids), structural support and water transport (polymeric lignins).

[004] Table 1 shows the two main groups of phenolic compounds, flavonoids and nonflavanoids, with some of the classes within each group. The flavonoids, are characterised by a C6-C3-C6 structure and are classified as by the type of heterocycle (e.g. flavonols, flavones, flavanones, flavanols, anthocyanidins and isoflavones) they contain. The non-flavonoids, are more varied and include simple phenols, hydroxybenzoic acids, hydroxycinnamic acids (HCAs), coumarins, and tannins, among others.

Table 1: Classification of the phenolic compounds

	Carbon skeleton	*Compound class*
Non-flavonoids	C_6	simple phenols
	C_6-C_1	hydroxybenzoic acids
	C_6-C_2	acetophenones, phenylacetates
	C_6-C_3	hydroxycinnamic acids, phenylpropenes, coumarins, chromones
	C_6-C_4	naphtoquinones
	C_6-C_1-C_6	xanthones
	C_6-C_2-C_6	stilbenes, anthraquinones
	$(C_6$-$C_3)_2$	lignins
	$(C_6)_n$	catechol melanins
	$(C_6$-$C_1)_n$:Glucose	hydrolysable tannins
	$(C_6$-$C_3)_n$	lignins
Flavonoids	C_6-C_3-C_6	flavonols
	C_6-C_3-C_6	flavones
	C_6-C_3-C_6	flavanones
	C_6-C_3-C_6	flavanols
	C_6-C_3-C_6	anthocyanidins
	C_6-C_3-C_6	isoflavones
	$(C_6$-C_3-$C_6)_2$	Biflavonoids
	$(C_6$-C_3-$C_6)_n$	condensed tannins (catechin polymers, proanthocyanidins)

[005] HCAs feature a unique C6-C3 chemical structure and are rarely found as free acids in unprocessed plant material. They are generally identified in plant extracts after degradation of the soluble and insoluble-bound derivatives (Clifford, 2000). HCAs possess a phenol group and a carboxylic acid function, characteristic of the phenolic acids including benzoic and cinnamic acid derivatives. Both *cis* and *trans* forms of cinnamic acid compounds are common in nature, the latter being naturally derived from the biosynthetic precursor t-cinnamic acid. Isomerisation to *cis* derivatives may occur during extraction, processing, or exposure to light (Kahnt, 1967). The simple benzoic and cinnamic acid derivatives differ in the degree of hydroxylation and/ or methoxylation of the aromatic ring (Table 2). Coumaric, caffeic, ferulic and sinapic acids are amongst the most widely distributed phenolic compounds in plants. Three position isomers exist for coumaric acid: 2-, 3- and 4-coumaric acids, with the latter occurring most abundantly in nature.

Table 2: Substitution of aromatic ring in phenolic compounds

Benzoic acids (C_6-C_1)	*Aromatic substitution*	Cinnamic acids (C_6-C_3)
salicylic acid	2-OH	2-coumaric acid
4-hydroxybenzoic acid	4-OH	(4-)coumaric acid
vanillic acid	3-OCH_3, 4-OH	ferulic acid
syringic acid	3,5-di-OCH_3, 4-OH	sinapic acid
-	3-OCH_3, 4-OH, 5-OH	5-hydroxyferulic acid
protocatechuic acid	3,4-di-OH	caffeic acid
gentisic acid	2,5-di-OH	-
gallic acid	3,4,5-tri-OH	-

[006] Other than gallic acid and its derivatives, all major groups of plant phenolics are synthesised by further metabolism of the simple HCAs. In many plants, hydroxycinnamoyl-CoA thioesters are the most common activated intermediates for phenylpropanoid biosynthesis (Ulbrich et al., 1979). Various hydroxycinnamoyl-CoA thioesters enter downstream pathways by taking part in different types of side-chain reactions including:

(a) side-chain elongation (condensation) with malonyl-CoA molecules leading to flavonoids, stilbenes, styrylpyrones, benzophenones, lignans and chain-elongated HCAs;

(b) NADPH-dependent reduction leading to dihydrocinnamic acids, hydroxycinnamyl alcohols (e.g. monolignols, precursors for lignins) and hydroxycinnamyl aldehydes;

(c) oxidative reactions resulting in side-chain shortening leading to benzoic acids;

(d) 2-hydroxylation and lactonisation leading to hydroxycoumarins; and

(e) conjugation with hydroxyacids or amino compounds leading to ester (e.g. CGAs) and amide conjugates, respectively.

[007] A schematic illustration of the reactions from which varied phenolic compounds are derived is presented in Figure 1.

[008] Chlorogenic acids (CGAs) are phenolic compounds which play a major role in the response to various biotic and abiotic stresses. CGAs are also important for their role as antioxidants in the diet of animals. Certain plants, such as coffee are rich in CGAs, and are among the most important dietary sources of this group of antioxidants. Hydroxycinnamic acids are commonly found as soluble conjugates, esters, amides or glycosides, within the cytoplasm of plant cells. Among these conjugates, CGAs have been shown to account for up to 90 % of the

total phenolic fraction of some plant species (see below). The generic name "CGA" used to refer to the single compound 5-O-caffeoylquinic acid (5-CQA), which was first detected in green coffee beans by Robiquet and Boutron in 1837 (Sondheimer, 1964). The definition was later extended to include all esters of quinic acid with a cinnamic acid derivative (Clifford, 2000). Quinic acid (1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid) is an alicyclic acid containing four readily accessible hydroxyl groups. CGAs are often grouped with esters of shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid), which differ from quinic acid by the presence of a double bond at C-1 and only three readily accessible hydroxyl groups. CGAs are generally classified depending on the identity, number and position of the acyl group (Clifford, 1999; Clifford, 2000). The common CGA groups are caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs) and dicaffeoylquinic acids (diCQAs), the chemical structures of which are known to the skilled artisan.

Table 3: Quinic acid and representative chlorogenic acid (CGA) species

Name	***Abbreviation***	***Substitution***			
		C-1	**C-3**	**C-4**	**C-5**
quinic acid	QA	OH	OH	OH	OH
3-*O*-caffeoylquinic acid	3-CQA	OH	C	OH	OH
4-*O*-caffeoylquinic acid	4-CQA	OH	OH	C	OH
5-*O*-caffeoylquinic acid (1)	5-CQA	OH	OH	OH	C
1,3-di-*O*-caffeoylquinic acid	1,3-diCQA	C	C	OH	OH
1,5-di-*O*-caffeoylquinic acid	1,5-diCQA	C	OH	OH	C
3,4-di-*O*-caffeoylquinic acid	3,4-diCQA	OH	C	C	OH
3,5-di-*O*-caffeoylquinic acid (2)	3,5-diCQA	OH	C	OH	C
4,5-di-*O*-caffeoylquinic acid	4,5-diCQA	OH	OH	C	C
5-*O*-feruloylquinic acid	5-FQA	OH	OH	OH	F

C = caffeic acid; F = ferulic acid

[009] Chlorogenic acids are formed between t-cinnamic acid derivatives and quinic acid., and are important intermediates for lignin biosynthesis in higher plants. CGA derivatives are abundant and diverse in the plant kingdom (Clifford, 1999; Clifford, 2000). They have been reported in several orders of mono- and dicotyledonous angiosperm species (Petersen *et al.*, 2009). The phylogenetically related *Rubiaceae* (e.g. plums, coffee), *Solanaceae* (e.g. tomato, tobacco, potato, eggplant), *Asteraceae* (e.g. artichoke, sunflower, chicory) and the more distant *Rosaceae* (e.g. apple, pears, peach) families possess most of the CGA-accumulating species.

Monoesters of caffeic acid are relatively widespread, with 5-CQA being by far the most predominant isomer in green coffee beans (Clifford, 1979; Aerts *et al.*, 1994), potato tubers (Griffiths *et al.*, 1997), tomato leaves (Niggeweg *et al.*, 2004) and tobacco leaves (Hoffmann *et al.*, 2004). Some plant families such as *Solanaceae* and *Rubiaceae* also abound in diesters, such as diCQAs, diFQAs and mixed diesters (e.g. caffeoylferuloylquinic acids). Many plants, including coffee, produce CGAs derivatives esterified at C-3, C-4 and C-5, but not at C-1 of the quinic acid moiety. This is in contrast with artichoke and some other *Asteraceae* plants, which abound in 1,3-diCQA (cynarin) and 1,5-diCQA, and also produce tri- and tetracaffeoylquinic acids.

[0010] Coffee plants contain the most diverse and highest amounts of CGAs reported thus far. *C. arabica* and *C. canephora* are the two *Coffea* species used extensively in the beverages known commercially as Arabica and Robusta coffee respectively. A total of 45 different CGAs have been identified in green Arabica coffee beans (Clifford *et al.*, 2006), while 69 CGAs have been reported in green Robusta coffee beans (Jaiswal, 2010). Quantitatively, the major coffee CGAs are the monocaffeoylquinic acids (3-CQA, 4-CQA and 5-CQA), the dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA and 4,5-diCQA) and the monoferuloylquinic acids (mainly 5-FQA). 5-CQA is the most abundant soluble ester, representing 45 to 50 % of the total CGAs in *C. canephora* seeds (Koshiro *et al.*, 2007). Other CGA compounds, such as coumaroylquinic and sinapoylquinic acids, as well as mixed diesters (e.g. caffeoylferuloylquinic acids) and 3,4,5-trimethoxycinnamoylquinic acid, can be found in low levels in Robusta coffee (Clifford, 2000; Jaiswal, 2010). *C. canephora* produces substantially more CGAs than *C. Arabica*.

[0011] The CGA level in green coffee grains varies between 7.9 and 14.4 % dry weight (DW) in *C. canephora* and 3.4 to 4.8 % DW in *C. arabica* (Ky *et al.*, 2001). (Lepelley *et al.*, 2007) reported lower but still relatively high CGA levels in *C. canephora* (6.6 % to 7.5 % DW). The content of diCQAs in *C. canephora* is also much higher than in *C. arabica*. CGA pools can vary with the coffee grain development (De Castro *et al.*, 2006; Lepelley *et al.*, 2007). While 5-CQA is the predominant molecule at all stages, 3-CQA and 4-CQA levels increase with seed growth. The 5-FQA level is relatively low in the early stage of grain development but rises 5 to 10 fold as the development progresses and reaches 22 % of total CGAs in ripe *C. canephora*.

3,5-diCQA is the major diCQA, especially in the early stages of fruit growth, but its level declines significantly during the development process.

[0012] Quinic acid is an abundant metabolite in young coffee grains, representing between 6 and 16 % DW. So, supply of this precursor may not restrict CGA biosynthesis as the grain develops. However, towards the end of grain development, quinic acid levels decrease below 1 % DW (Rogers *et al.*, 1999; Lepelley *et al.*, 2007). Considerable amounts of inositol (3-4 % DW) are found in young coffee grains and other organic acids, such as citric and malic acids, are dominant in the mature coffee grain (Rogers *et al.*, 1999). In Robusta coffee, caffeic and coumaric acids have also been found as conjugates of tryptophan (Murata *et al.*, 1995).

[0013] Shikimate esters (also named dactylifric acids) have been reported in *Palmae* plants (e.g. palm, date, endive) (Maier *et al.*, 1964; Harborne *et al.*, 1974; Goupy *et al.*, 1990; Ziouti *et al.*, 1996). The shikimic acid precursor fails to accumulate to measurable levels in most of the important agronomic crops, although it is found in star anise (*Illicium verum*) and various species of evergreen trees. An ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, commonly known as rosmarinic acid, is one of the active components of several medicinal plants and predominant in some *Boraginaceae* and *Lamiaceae* species, such as basil (*Coleus blumei*) and rosemary (*Rosmarinus officinalis*) (Petersen *et al.*, 2003; Abdullah *et al.*, 2008; Petersen *et al.*, 2009). HCAs may also be conjugated to other acyl acceptors such as aliphatic acids (e.g. acetic, citric, malic, glucaric, tartric acids), sugar alcohols (e.g. arabinose, galactose, glucose, rhamnose), amino compounds (e.g. tryptophan, tyramine, octopamine, anthranilate, agmatine, choline, spermidine) and lipids (Clifford, 2000). The leaves of red clover (*Trifolium pratense*) accumulate phaselic acid (2-*O*-caffeoyl-L-malic acid), which prevents the breakdown of forage proteins during storage (Sullivan, 2009). *Arabidopsis thaliana* and other members of the *Brassicaceae*, such as radish (*Raphanus sativus*), accumulate hydroxycinnamoyl esters formed with aliphatic acids. These include sinapoyl-malic acid, a leaf-specific ester, as well as sinapoyl-choline, a seed-specific ester, and their common biosynthetic precursor sinapoyl-glucose (Chapple *et al.*, 1992).

[0014] Besides the importance for the plant itself, HCAs and CGAs have attracted focus because they can influence food properties such as colour, taste and aroma. In general, phenolic compounds are considered to negatively influence food selection as they impart a bitter flavour (Drewnowski, 1997). CGAs, especially the diesters of caffeic acid, constitute a class of

astringent compounds (Ohiokpehai *et al.*, 1983). In coffee beverages, (Farah *et al.*, 2006) reported that 3,4-diCQA levels in green and roasted coffee strongly correlate with satisfactory sensory attributes, but that higher levels of CQAs and 5-FQA are detrimental to beverage quality. A higher CGA content could be responsible for the perceived inferior quality of brews made with Robusta, which are occasionally described as bitter (Bertrand *et al.*, 2003).

[0015] CGAs and other HCA derivatives are significant components of diets rich in plant-based food such as cereals, legumes, oilseeds, fruits, vegetables and beverages. An increasing number of epidemiological studies have shown that people who consume higher quantities of plant-based food appear to have lower risks for significant health problems, such as cardiovascular diseases and certain cancers (Clifford, 2004; Finley, 2005). This health-promoting effect has been associated with the presence of phenolic phytonutrients. When compared to other beverages, coffee represents the richest source of CGAs and, interestingly, it has the highest antioxidant activity (Clifford, 1999; Wang *et al.*, 2009). The presence of conjugated ring structures and hydroxyl groups allows CGAs to actively scavenge free radicals generated in the aqueous phase (Rice-Evans *et al.*, 1996). This ability can prevent oxidative damage and lipid peroxidation in living tissues. While caffeic acid and 5-CQA have similar antioxidant capacities *in vitro* (Rice-Evans *et al.*, 1996), the number and nature of substitutions on the aromatic ring can influence the antioxidant properties. For instance, it has been shown *in vitro* that hydroxyl groups are more electron donating than methoxyl groups and that dihydroxyl-substituted caffeic acid shows a higher antioxidant activity than mono-substituted coumaric acid (Foley *et al.*, 1999; Shahidi *et al.*, 2010). Further studies demonstrate that CGAs can exert their health benefits through various other mechanisms, such as metal chelation and modification of redox potentials (Rice-Evans *et al.*, 1996).

[0016] CGAs have attracted much attention due to their exceptional antioxidant properties, as well as their high bioavailability and absorption in humans (Nardini *et al.*, 2002). Following coffee intake, CGAs are absorbed directly by the small intestine or hydrolysed by the large intestine microflora to release caffeic acid (Nardini *et al.*, 2002; Stalmach *et al.*, 2009; Stalmach *et al.*, 2010). This degradation product, which has same antioxidant capacities as 5-CQA *in vitro* (see above), is also absorbed and relatively stable in the gut (Scalbert *et al.*, 2002). CGAs have been attributed many other health benefits including anti-inflammatory, antibacterial, antiproliferative and anticarcinogenic properties (Rice-Evans *et al.*, 1997). CGAs have also been

associated with reduced hepatic glucogenolysis and glucose absorption (Johnston *et al.*, 2003), inhibition of HIV-1 integrase (McDougall *et al.*, 1998), caffeine antagonistic cerebral effects (de Paulis *et al.*, 2004), reduced incidence of atherosclerosis, type 2 diabetes and various types of cancer (McCarty, 2005; Natella *et al.*, 2007). CGAs have been proposed to play a role in the prevention of neuro-degenerative diseases (Shahidi *et al.*, 2010).

[0017] Because CGAs and the activated CoA-thioesters needed to make them are difficult to synthesize, and nearly impossible to make in commercially useful quantities, there is a need for methods of producing chlorogenic and dactylifric acid species, as well as activated CoA-thioesters *in vitro*.

SUMMARY OF THE INVENTION

[0018] One aspect of the present invention features a recombinant *Coffea* wild-type HCT, HQT, and/or 4CL enzyme produced in a microbial cell and having enzyme activity substantially identical to a corresponding enzyme isolated from a *Coffea spp.* plant. Various alternative, independently selected embodiments of this aspect of the invention include the following: (1) the *Coffea* plant is *C. canephora*; (2) the microbial cell is *E. coli*, *S. cerevisiae*, or a food-grade lactic acid bacteria; (3) the recombinant enzyme is purified substantially to homogeneity; (4) the recombinant enzyme is immobilised on or covalently attached to a matrix; (5) the gene encoding the recombinant enzyme is engineered to provide more optimum codon usage for the microorganism in which it is to be expressed; (6) the gene encoding the recombinant enzyme is engineered to provide a purification tag on the N-terminus of the recombinant enzyme; (7) the enzyme is a 4CL enzyme capable of catalysing the production of a hydroxycinnamic acid (HCA)-CoA thioester from an HCA in the presence of coenzyme A (CoA), a divalent metal ion, and adenosine triphosphate (ATP); (8) the recombinant enzyme is catalytically active *in vivo* in the microorganism, or *in vitro*; (9) the recombinant enzyme catalyses the production of a CoA thioester from at least cinnamic acid, coumaric acid, caffeic acid, ferulic acid, and sinapic acid; (10) the recombinant enzyme is capable of converting at least about 80% of cinnamic acid, coumaric acid, caffeic acid, or ferulic acid to the corresponding HCA-CoA; (11) the recombinant enzyme is capable of producing *in vitro* a HCA-CoA thioester substantially free from the HCA acid precursor; (12) the recombinant enzyme is a HCT or HQT enzyme capable of catalysing *in vivo* in the microorganism or *in vitro* the production of a chlorogenic acid species or dactylifric

acid species from a HCA-CoA thioester and an acyl acceptor molecule, and in particular wherein the HCA-CoA thioester comprises one or more of the thioesters coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA; and further wherein the acyl acceptor comprises quinic acid, shikimic acid, or a mixture thereof; (13) if the enzyme is recombinant HCT, it has a preference for shikimic acid as the acyl acceptor; (14) if the enzyme is recombinant HQT, it has a preference for quinic acid as the acyl acceptor; (15) if the enzyme is recombinant HCT or HQT, it is capable of catalysing the production *in vitro* of a HCA-CoA thioester from a chlorogenic acid or related ester in the presence of CoA in the temperature range of at least 22-42 °C; (16) the recombinant enzyme is HCT and the HCA-CoA thioester formed is a di-, tri- or higher CGA or related ester; and (17) the recombinant enzyme has an amino acid of SEQ ID NO: 23 or 44 or 47.

[0019] Another aspect of the present invention features a mutant plant HCT enzyme comprising an amino acid sequence that is at least 80% identical to that of a corresponding wild-type HCT enzyme found in the same species of plant, and contains an altered amino acid residue in one or more positions relative to the wild-type HCT enzyme, wherein the mutant can bind at least one substrate or catalyze the formation of at least one product in either the forward or reverse direction. In various embodiments, the altered amino acid residue is at a position corresponding to an amino acid in a reference amino acid sequence that is the *Coffea canephora* HCT enzyme sequence (SEQ ID NO:23) and the amino acid is W23, N26, V27, D28, L29, V30, V31, P32, N33, F34, H35, T36, P37, S38, V39, Y40, P110, R115, G143, G144, V149, G150, M151, R152, H153, H154, A155, A156, D157, G158, F159, S160, G161, L162, H163, F164, I165, Y203, K210, K217, L231, N248, Y252, Y255, L272, V274, D275, Q276, L280, Y281, I282, A283, T284, D285, R289, L294, N301, V302, I303, F304, T305, L331, L346, K353, L355, V356, R357, G358, A359, H360, T361, F362, K363, C364, P365, N366, L367, G368, I369, T370, S371, W372, V373, R374, L375, P376, I377, G383, M390, G391, P392, G393, G394, I395, A396, Y397, E398, G399, L400, S401, or F402, or any combinations thereof. The alteration is a conservative substitution in one embodiment. In certain embodiments, the mutant HCT has improved ability relative to the wild-type HCT to bind at least one substrate, or to catalyze the formation of at least one product in either the forward or backward direction, or greater resistance to proteolysis. The mutant HCT may show a substrate preference for shikimic acid species relative to the wild-type enzyme. The mutant HCT may also or alternatively show

an improved ability to catalyze the formation of diCQA relative to the wild-type enzyme. In certain embodiments, the mutant HCT comprises an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HCT sequence (SEQ ID NO:23). It may comprise one or more of the mutations H35A, H153A, H154A, A155L, A156S, D157A, Y252A, Y255A, R374E, K210A/K217A, or H154N/A155L/A156S. The mutant HCT can be a mutant of *C. canephora* wild-type HCT enzyme. In certain embodiments, it has an amino acid sequence that is any of SEQ ID NOs:25, 27, 29, 31, 33, 35, 37, 39, or 41.

[0020] Another aspect of the invention features a mutant plant HQT enzyme comprising an amino acid sequence that is at least 80% identical to that of a corresponding wild-type HQT enzyme found in a plant, but which contains an altered amino acid residue in one or more positions relative to the wild-type HQT enzyme, wherein the mutant can bind at least one substrate or catalyze the formation of at least one product in either the forward or reverse direction. In various embodiments, the altered amino acid residue is at a position corresponding to an amino acid in a reference amino acid sequence that is the *Coffea canephora* HQT enzyme sequence (SEQ ID NO:44) and the amino acid is W23, N26, I27, D28, L29, L30, V31, A32, R33, I34, H35, I36, L37, T38, V39, Y40, P110, R115, G143, A144, A149, G150, V151, Q152, H153, N154, L155, S156, D157, G158, V159, S160, S161, L162, H163, F164, I165, Y203, K210, L217, I231, N245, E246, G247, Y252, L272, N274, D275, Q276, L280, Y281, V282, A283, T284, D285, R289, L294, N301, V302, I303, F304, T305, L331, L346, S353, L355, V356, R357, G358, D359, R360, H361, F362, A363, S364, P365, N366, L367, N368, I369, N370, S371, W372, T373, R374, L375, P376, F377, G383, I390, G391, P392, A393, I394, I395, L396, Y397, E398, G399, T400, V401, or Y402. The alteration can be a conservative substitution. In certain embodiments, the mutant HQT has improved ability relative to the wild-type HQT to bind at least one substrate, or to catalyze the formation of at least one product in either the forward or backward direction, or greater resistance to proteolysis. The mutant HQT can show a substrate preference for quinic acid species relative to the wild-type enzyme. It can also or alternatively show an improved ability to catalyze the formation of diCQA relative to the wild-type enzyme. In certain embodiments, the mutant HQT comprises an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HQT sequence (SEQ ID NO:44). It may comprise one or more of the

mutations H35A, H153A, N154A, L155A, S156A, D157A, Y252A, R374E, K210A, or combinations thereof. The mutant HQT can be a mutant of *C. canephora* wild-type HQT enzyme. In one embodiment, it has an amino acid sequence that is SEQ ID NO: 46.

[0021] Another aspect of the invention features a composition comprising one or more of the above-described recombinant enzymes or mutants thereof, adapted for *in vitro* synthesis of one or more chlorogenic acid species or dactylifric acid species. The composition can further comprise substrates and cofactors sufficient for the production of one or more chlorogenic acid species or dactylifric acid species. In certain embodiments, one or more of the enzymes is immobilised on or covalently bound to a matrix. In certain embodiments, the substrates comprise one or more of coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA, and an acyl acceptor, the cofactors comprise Mg^{++}, ATP, and optionally, CoA. The acyl acceptor can comprise quinic or shikimic acid species. In particular embodiments, the chlorogenic acid species or dactylifric acid species comprise caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs), caffeoylshikimic acids (CSAa), coumaroylshikimic acids, feruloylshikimic acids (FSAs), dicaffeoylquinic acids (diCQAs), or other di-, tri- or tetraesters, or combinations of any of the foregoing. The composition can be adapted for *in vitro* production of a HCA-CoA thioester catalysed by a 4CL enzyme, prior to *in vitro* production of the chlorogenic acid species or dactylifric acid species. In particular, the substrates for production of the HCA-CoA thioester can comprise one or more of cinnamic acid, coumaric acid, caffeic acid, or ferulic acid, and CoA.

[0022] Another aspect of the invention features a method of producing a chlorogenic acid or dactylifric acid species *in vitro* comprising the steps of: (a) providing an HCT or HQT or both; b) providing substrates and cofactors sufficient to form a chlorogenic acid or dactylifric acid species in the presence of an HCT or HQT or both; and (c) contacting the substrates and cofactors with the HCT or HQT, or both, under conditions permitting enzymatic activity, for a time sufficient to permit the formation of product that is a chlorogenic acid or dactylifric acid species. In certain embodiments, the substrates comprise a hydroxycinnamoyl-CoA thioester, and an acyl acceptor. In particular, the acyl acceptors include quinic acid, shikimic acid, or a combination thereof. In additional or alternative embodiments, the substrates comprise a mixture of coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA. In certain embodiments, the product comprises a mono-, di-, tri- or tetraester species of chlorogenic or

dactylifric acid. In other embodiments of the method, the chlorogenic acid or dactylifric species comprise caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs), caffeoylshikimic acida (CSAa), coumaroylshikimic acids, feruloylshikimic acids (FSAs), dicaffeoylquinic acids (diCQAs), or other di-, tri- or tetraesters, or combinations of any of the foregoing. The method may further comprise an additional step wherein a HCA-CoA thioester is produced using a 4CL enzyme. In various embodiments of the method, the substrates comprise CoA, and one or more chlorogenic acid or dactylifric acid species. At least one of the HCT or HQT can be immobilised on or covalently bound to a matrix. The HCT or the HQT can be a *Coffea* HCT or HQT.

[0023] Another aspect of the invention features a method of producing a chlorogenic acid or dactylifric acid species *in vitro* comprising the steps of (a) providing an HCT, and optionally, an HQT or 4CL or both; (b) providing one or more HCA thioesters, an acyl acceptor, CoA, and a first chlorogenic acid or dactylifric acid species; and (c) contacting the one or more HCA thioesters, acyl acceptor, CoA, and first chlorogenic acid or dactylifric acid species with the HCT under conditions permitting enzymatic activity, and for a time sufficient to permit the formation of product that is a second chlorogenic acid or dactylifric acid species. In one embodiment, the first chlorogenic acid or dactylifric acid species is catalytically converted into a second chlorogenic acid or dactylifric acid species.

[0024] Another aspect of the invention features a food product comprising one or more exogenous chlorogenic acid or dactylifric acid species. The chlorogenic acid or dactylifric acid species in the food product can be produced *in vitro*. The exogenous chlorogenic acid or dactylifric acid species can be added to the food product as a mixture to the food product during processing. In one embodiment, the food product is a coffee product. The coffe product can be a soluble coffee product in which the exogenous chlorogenic acid or dactylifric acid species provide a functional ingredient that improves quality or provides a health benefit. In various embodiments, the exogenous chlorogenic acid or dactylifric acid species directly or indirectly provide a desirable flavour attribute, aroma attribute, or colour attribute to the product. Alternatively or additionally, the exogenous chlorogenic acid or dactylifric acid species can contribute antioxidant properties to the product. The exogenous chlorogenic acid or dactylifric acid species in the food product may comprise mono- and diester CGA or CSA species. In particular, the exogenous chlorogenic acid can comprise 3,4, caffeoylquinic acid at levels that

improve the flavour and aroma of the food product as determined by sensory evaluation. In a specific instance, the exogenous chlorogenic acid comprises 3,4, caffeoylquinic acid but is substantially free of 5-feruloylquinic acid.

[0025] Another aspect of the invention features a recombinant microorganism comprising a gene for an expressible exogenous enzyme that is a *Coffea* HCT or HQT; wherein the microorganism expresses the enzyme. The recombinant microorganism can be a food-grade lactic acid bacterium. In certain embodiments, the recombinant microorganism produces a chlorogenic acid or dactylifric acid species in an amount not naturally produced in a comparable microorganism that does not express the enzyme.

[0026] Another aspect of the invention features a transgenic plant comprising a heterologous gene for an expressible exogenous enzyme that is a *Coffea* HCT or HQT; wherein the plant expresses the enzyme. In one embodiment, the transgenic plant produces less lignin than an equivalent plant that does not comprise the heterologous gene. In an additional or alternative embodiment, the transgenic plant comprises substantially equivalent biomass and growth characteristics as an equivalent plant that does not comprise the heterologous gene, under equivalent growth conditions.

[0027] Other and further objects, features, and advantages of the present invention will be readily apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] Fig. 1: Side-chain reactions involving hydroxycinnamoyl-CoA thioesters (Adapted from Strack *et al.*, 1997).

[0029] Fig. 2: SDS-PAGE (Panel A) and western-blot (Panel B) analysis of *Cc*4CL2 following over-expression and purification. Lanes 1-6 as follows: Lane 1: not induced; lane 2: induced; lane 3: insoluble cell extract; lane 4: soluble cell extract; lane 5: flow through; lane 6: elution fraction; M: MW marker (kDa).

[0030] Fig. 3: SDS-PAGE analysis of the soluble fraction of the cell lysates following *Nt*4CL2 over-expression. Lanes 1-6 are as follows: Lane 1: BL21 (DE3); lane 2: BL21* (DE3); lane 3: BL21 (DE3) pLysS; lane 4: BL21* (DE3) pLysS; M: MW marker (kDa).

[0031] Fig. 4: SDS-PAGE analysis of Nt4CL2 following purification by size-exclusion chromatography. Panel (A): Chromatogram. Panel (B): 12 % SDS-PAGE; lane 1: purified Nt4CL2; M: MW marker (kDa).

[0032] Fig. 5: Analysis of GST-HQT following expression and affinity chromatography. Panel (A): 12 % SDS-PAGE. Panel (B): Western-blot against the GST-fusion. M: MW marker (kDa); Lanes were as follows: lanes 1-5: not induced, induced, lysate, soluble extracts 1 and 2; lanes 5-18: wash and elution fractions from the affinity column.

[0033] Fig. 6: Agarose gel analysis of PCR products from *Cc*hct and *Cc*hqt amplification. Panel (A): Amplification of *Cc*hct. Panel (B): Amplification of *Cc*hqt. Lanes 1, 2, 3, 4: High Fidelity enzyme mix; lanes 5, 6, 7, 8: Pfu polymerase; M: MW marker.

[0034] Fig. 7: SDS-PAGE and western-blot of His6-CcHCT over-expression in BL21* (DE3) pLysS cells. Panel (A): 12 % SDS-PAGE. Panel (B): Western-blot against the His6-tag. Lanes were as follows: 1: non-induced cells; 2: soluble cell extract; 3: insoluble cell extract; 4: flow through; 5: wash; 6: elution; M: MW marker.

[0035] Fig. 8: Chromatogram and SDS-PAGE analysis of CcHCT from size-exclusion chromatography. Panel (A): Chromatogram for the size-exclusion step. Panel (B): 12% SDS-PAGE. Lanes were as follows: 1: injected sample, M: MW marker (kDa); lanes 2-14: collected fractions n° 19-25 in the reverse order.

[0036] Fig. 9: SDS-PAGE analysis of HCT proteolysis. Panel (A): Lane 1: purified *Cc*HCT stored at -80 °C. M: MW marker (kDa); lane 2: same sample stored at 4 °C for several weeks. Panel (B): Limited proteolysis of with trypsin at a 1/500 (w/w) dilution on ice. M: MW marker; t0-t60: incubation time in min. (C) Limited proteolysis of the methylated derivative of *Cc*HCT.

[0037] Fig. 10: SDS-PAGE analysis of the K-mutant HCT following limited proteolysis. Lanes were as follows: M: MW marker (kDa), lanes 1-5: trypsin digestion, lanes 6-10: chymotrypsin digestion, lanes 11-15: subtilisin digestion, respectively at t= 1, 5, 15, 60 min and overnight incubation with 1/1000 (w/w) protease at 4 °C.

[0038] Fig. 11: SDS-PAGE analysis of His6-SUMO3-*Cc*HQT during the purification procedure. Panel (A): Lanes 1-4: cell lysate; lanes 5-8: fractions from the first affinity column, lanes 9-10: before and after SenP2 digestion, lanes 11-16: fractions from the ion-exchange column, M: MW marker (kDa). The band corresponding to His6-SUMO3-HQT/ cleaved HQT is

circled in red. Panel (B): size-exclusion chromatogram. Panel (C): Degradation pattern of *Cc*HQT after storage at 4° C for several weeks.

[0039] Fig. 12: Overlap of the absorption spectra of CoA, 5-CQA and the reaction product caffeoyl-CoA. Reactions were set up using 5 mM CoA and 5 mM 5-CQA in 0.1 M sodium phosphate pH 6.0. The reaction was started by adding 0.002 µM HQT and incubated at 34 °C. Samples taken at t= 0 (Panel A) and 30 min (Panel B) were analysed by HPLC with photodiode array (PDA) detector.

[0040] Fig. 13: HPLC analysis of reactions comprising Nt4CL2 incubated with (hydroxy)cinnamic acids and CoA. Reactions were set up with 0.1 M Tris-HCl pH 7.5, 5 mM CoA and 1 mM of the various (hydroxy)cinnamic acids noted in each panel. Reactions were started by adding 1.6 µM of enzyme. Samples taken at the times indicated were analysed using HPLC-PDA with the methanol method. All chromatograms were extracted at λ= 325 nm except for cinnamic acid (Panel A), which was measured at λ= 300 nm. Panel (A): Cinnamic acid at t= 0 min (sample 598) and t= 20 min (sample 600); Panel (B) Coumaric acid at t= 0 min (sample 593) and t= 20 min (sample 594); Panel (C) Caffeic acid at t= 0 min (sample 590) and t= 20 min (sample 592); Panel (D) Ferulic acid at t= 0 min (sample 595) and t= 20 min (sample 597); and (E) Sinapic acid at t= 0 min (sample 601) and t= 20 min (sample 603).

[0041] Fig. 14: Absorption spectra of the CoA thioester products measured using HPLC-PDA. Panel (A): cinnamoyl-CoA; Panel (B) coumaroyl-CoA; Panel (C) caffeoyl-CoA; Panel (D) feruloyl-CoA; and Panel (E) sinapoyl-CoA.

[0042] Fig. 15: CoA thioester production by recombinant *Nt*4CL2 with various HCAs and CoA in the presence of Mg-ATP. At t= 0, Enzyme (0.05 µM *Nt*4CL2) was added to a 10 mL reaction mixture containing 0.8 mM coumaric/ caffeic or ferulic acid, 1 mM CoA, 2 mM MgCl2 and 2 mM ATP in 0.1 M sodium phosphate pH 6.0. Samples (699-715) were taken at t= 0, 10, 60 and 150 min and analysed using HPLC-PDA with the methanol method. The conversion to CoA thioester in percentage was calculated from the HCA precursor peak area diminution.

[0043] Fig. 16: A representative reaction scheme showing forward and reverse reactions catalysed by HCT and/or HQT.

[0044] Fig. 17: HPLC profiles of HCT reactions with caffeoyl-CoA and quinic/ shikimic acid. Reactions were set up with 0.1 M sodium phosphate pH 6.5, 0.2 mM caffeoyl-CoA and 5

mM quinic / shikimic acid, or a mixture. Reactions were started by adding 1 µM enzyme. Samples taken at t= 5 and 60 min were analysed using HPLC-PDA with the methanol method. An overlap of the absorbance spectra for the putative CSA isomers is indicated in panel D (sample 1042). Panel (A): caffeoyl-CoA, t= 0 min (sample 1023); Panel(B): Quinic acid, HCT t= 60 min (sample 1041); Panel (C): Shikimic acid, HCT t= 5 min (sample 1027); Panel (D): Shikimic acid, HCT t= 60 min (sample 1042); Panel (E): Quinic and shikimic acids, HCT t= 5 min (sample 1028); and Panel (F): Quinic and shikimic acids, HCT t= 60 min (sample 1043).

[0045] Fig. 18: HPLC profiles of HQT reactions with caffeoyl-CoA and quinic/ shikimic acid. Reactions were set up with 0.1 M sodium phosphate pH 6.5, 0.2 mM caffeoyl-CoA and 5 mM quinic/ shikimic acid or both. Reactions were started by adding 1 µM enzyme. Samples taken at t= 5 and 60 min were analysed using HPLC-PDA with the methanol method. See Fig. 17 Panel (A) for the control reaction. Panel (A): Quinic acid, HQT t= 60 min (sample 1044); Panel (B): Shikimic acid, HQT t= 60 min (sample 1045); Panel (C): Quinic and shikimic acids, HQT t= 5 min (sample 1031); and Panel (D): Quinic and shikimic acids, HQT t= 60 min (sample 1046).

[0046] Fig. 19: HPLC profiles of HCT reactions with coumaroyl-CoA and quinic/ shikimic acid. Reactions were set up with 0.2 mM coumaroyl-CoA and 10 mM quinic/ shikimic acid/ or a mixture of both in 0.1 M sodium phosphate pH 6.0. Reactions were started by adding 0.4 µM enzyme. Samples taken at t= 10 min were analysed using HPLC-PDA with the methanol method. Panel (A): coumaroyl-CoA, t= 0; Panel (B): Quinic acid, t= 10 min (sample 764); Panel (C): Shikimic acid, t= 10 min (sample 765); and Panel (D): Quinic and shikimic acids, t= 10 min (sample 766).

[0047] Fig. 20: HPLC profiles of HQT reactions with coumaroyl-CoA and quinic/ shikimic acid. The same conditions as Fig. 19 were applied. Panel (A): Quinic acid, t= 10 min (sample 767); Panel (B): Shikimic acid, t= 10 min (sample 768); and Panel (C): Quinic and shikimic acids, t= 10 min (sample 769).

[0048] Fig. 21: HPLC profile of HCT reactions with feruloyl-CoA and quinic/ shikimic acid. Reaction mixtures containing 0.2 mM feruloyl-CoA, 10 mM quinic or shikimic acid or both and 0.1M sodium phosphate pH 6.0 were prepared in a 100 µL final volume. The reaction was started by adding 0.01 µM HCT. Samples taken at t= 20 min were analysed using HPLC-PDA with the methanol method. Panel B shows the absorption spectrum of the putative

feruloylshikimic acid peak. Panel (A): feruloyl-CoA t= 0; Panel (B): Shikimic acid t= 2 min (sample 717); Panel (C) Quinic acid t= 20 min (sample 722); and Panel (D) Quinic and shikimic acids t= 2 min (sample 718).

[0049] Fig. 22: HPLC profiles of HQT reactions with feruloyl-CoA and quinic/ shikimic acid. The same conditions as presented in Figure 21 were applied. Panel (A): Quinic acid t= 2 min (sample 719); Panel (B): Shikimic acid t= 2 min (sample 720); and Panel (C): Quinic and shikimic acids t= 2 min (sample 721).

[0050] Fig. 23: HPLC profiles of CQA standards upon heating. Samples containing 1 mM CQA isomer and 0.1M sodium phosphate pH 6.5 were incubated overnight and analysed using HPLC-PDA with the acetonitrile method. Panel (A): 5-CQA t= 0 (sample 1371); Panel (B): 4-CQA t= 0 (sample 1370); Panel (C): 3-CQA t= 0 (sample 1369); Panel (A'): 5-CQA overnight at 34 °C (sample 1377); Panel (B'): 4-CQA overnight at 34 °C (sample 1376); and Panel (C'): 3-CQA overnight at 34 °C (sample 1375).

[0051] Fig. 24: HPLC profiles of HCT and HQT incubated with 5-CQA and CoA. Reactions were set up with 0.1 M sodium phosphate pH 6.0, 3.75 mM 5-CQA, 5 mM CoA, and milliQ. Reactions were started by adding 1 µM HCT or 1.0/ 0.01 µM HQT to the reaction mixture. Samples taken at t= 5, 15, 30 and 60 min were analysed using HPLC-PDA with the methanol method. The retention times here are different from earlier experiments due to the different chromatographic conditions. Panel (A): t= 0 (sample 427); Panel (B): 1 µM HCT t= 60 min (sample 425); Panel (C): 1 µM HQT t= 60 min (sample 419); and Panel (D): 0.01 µM HQT t= 60 min (sample 423).

[0052] Fig. 25: HPLC profiles of HCT and HQT incubated with 5-FQA and CoA. Reactions were set up with 0.1 M sodium phosphate pH 6.0, 3.75 mM 5-FQA, 5 mM CoA and milliQ water in a final volume of 100 µL. Reactions were started by adding 1 µM HCT/ HQT. Samples (395-428) taken at t= 0, 5, 15, 30 and 60 min were analysed using HPLC-PDA with the methanol method. Panel (A): t= 0 (sample 428); Panel (B): 1 µM HCT t= 60 min (sample 426); and Panel (C): 1 µM HQT t= 60 min (sample 420).

[0053] Fig. 26: HPLC profiles of CcHQT incubated with 3-, 4- and 5-CQA isomers and CoA. Reactions were set up with 1 mM CQA and 5 mM CoA in 0.1 M sodium phosphate pH 7.5. Reactions were started by adding 0.4 µM HQT. Samples taken at t= 0 and 30 min were analysed using HPLC-PDA with the methanol method. Panel (A): 3-CQA, t= 0 and t= 30 min

(sample 612); Panel (B): 4-CQA, t= 0 and t= 30 min (sample 611); and Panel (C) 5-CQA, t= 0 and t= 30 min (sample 610).

[0054] Fig. 27: HPLC profiles of HCT incubated with 3,5-diCQA and CoA. Reactions were set up containing 1 mM 3,5-diCQA and 5 mM CoA in 0.1 M sodium phosphate pH 6.0 to a final volume of 200 µL. Reactions were started by adding 1 µM HCT/ HQT/ water as a control. Panel (A): Standards: 5-CQA and 3,5-diCQA; Panel (B): Control with no enzyme overnight (sample 882); and Panel (C): HCT overnight (sample 880).

[0055] Fig. 28: Influence of pH on caffeoyl-CoA production by HCT and HQT. Reactions were set up with 1 µM enzyme, 2 mM CoA and 2 mM 5-CQA in 0.1 M sodium phosphate at pH 4.6/ 6.0/ 7.6. Reactions were stopped at t= 300 min.

[0056] Fig. 29: Influence of pH on HCT and HQT activity towards 5-CQA and CoA. Reactions were set up with 2 mM CoA and 2 mM 5-CQA in 0.1 M sodium phosphate at pH 6.0, 7.0 and 8.0. Reactions were started by adding 1 µM HCT (A) or 0.1 µM HQT (B).Panel (A): HCT; Panel (B): HQT. Symbols: Squares: pH 6.0; Triangles: pH 7.0; Plain line: pH 8.0.

[0057] Fig. 30: Caffeoyl-CoA formation as a function of enzyme concentration. Reactions were set up with 5 mM CoA and 2 mM 5-CQA in 0.1 M sodium phosphate pH 6.0. The reaction was started by adding enzyme either HCT (Panel A) or HQT (Panel B). Enzyme concentration units tested are expressed in nM (HCT: 135, 271, 542, 813 nM; HQT: 8, 16, 33, 65, 130, 260, 521 nM).

[0058] Fig. 31: Influence of 5-CQA concentration on caffeoyl-CoA formation catalysed by HQT. Reactions were set up with 0.08-10 mM 5-CQA and 2 mM CoA in 0.1 M sodium phosphate pH 6.0. Reactions were started by adding 0.1 µM HQT.

[0059] Fig. 32: Inhibitory effect of quinic acid on the level of caffeoyl-CoA formation by HQT. Reactions were set up with 5 mM 5-CQA, 5 mM CoA and 0-20 mM quinic acid in 0.1 M sodium phosphate pH 6.0. Reactions were started by adding 1 µM HQT. Samples taken at t= 0, 5 and 30 min (488-503) were analysed using HPLC-PDA with the methanol method.

[0060] Fig. 33: Influence of CoA concentration on the level of caffeoyl-CoA formed by HCT and HQT. Reactions were set up with 0.125-5 mM CoA and 1 mM 5-CQA in 0.1 M sodium phosphate pH 6.5. Reactions were started by adding 5 µM HCT or 0.5 µM HQT. Samples (1101-1145) taken at t= 0 and 180 min were analysed using HPLC-PDA with the acetonitrile method. Panel (A): Standard Curve for CoA concentration added; Panel (B): Effect

of CoA concentration on the level of caffeoyl-CoA formation catalysed by HCT and HQT. Symbols: Diamonds: HCT; Triangles: HQT.

[0061] Fig. 34: HPLC profiles of HCT/ HQT incubated with 5-CQA and CoA at two different molar ratios. Reactions were set up with 1 mM 5-CQA and either 1 or 10 mM CoA in 0.1 M sodium phosphate pH 6.5. Reactions were started by adding 0.5 µM enzyme. Samples taken at t= 180 min were analysed using HPLC-PDA with the acetonitrile method. Panel (A): t= 0; Panel (B): Control 5-CQA and CoA no enzyme overnight; Panel (C): HCT 1/1 5-CQA/CoA ratio (sample 1085) and overlap of absorbance spectra of the diCQA peaks; Panel (D): HCT 1/10 5-CQA/CoA ratio (sample 1086); Panel (E): HQT 1/1 5-CQA/CoA ratio (sample 1089); and Panel (F): HQT 1/10 5-CQA/CoA ratio (sample 1090).

[0062] Fig. 35: Comparison of the activities of native and mutant HCTs in the forward and reverse reactions. Reaction mixtures are described below. Reactions were started by adding 5 µM enzyme. Samples taken at t= 210 min were analysed using HPLC-PDA with the acetonitrile method. Panels A, B, C, D, E, F, G, H: Reverse reaction: Reactions were set up with 1 mM 5-CQA and 1 mM CoA in 0.1 M sodium phosphate pH 6.5. Panels A'-K': Forward reaction: Reactions were set up with 0.1 mM caffeoyl-CoA and 1 mM quinic acid in 0.1 M sodium phosphate pH 6.5. Panels A / A': Control with no enzyme (samples 1321 / 1337); Panels B / B': native HCT (samples 1322 / 1338); Panels C / C': K-mutant (samples 1323 / 1339); Panels D / D': H35A mutant (samples 1326 / 1342); Panels E / E': Y255A mutant (samples 1329 / 1345); Panels F / F': D157A mutant (samples 1325 / 1341); Panels G / G': Y252A mutant (samples 1327 / 1343); Panels H / H': R374E mutant (samples 1328 / 1344); Panels I and I': H153A mutant (samples 1324 / 1340); Panels J and J': H154A mutant (samples 1330 / 1346); and Panels K / K': H154N/A155L/A156S mutant (samples 1331 / 1347).

[0063] Fig. 36: HPLC profiles of HCT H154 mutants incubated with 5-CQA and CoA at 1/1 and 1/10 molar ratios. Reactions were set up with 1 mM 5-CQA and 1 or 10 mM CoA in 0.1 M sodium phosphate pH 6.5. Reactions were started by adding 0.5 µM enzyme. Samples taken at t= 180 min were analysed using HPLC-PDA with the acetonitrile method. The t= 0 is the same as in Fig. 32. Panel (A): H154A 1/1 5-CQA/CoA (sample 1093); Panel B) H154A 1/10 5-CQA/CoA (sample 1094); Panel (C) H154N/A155L/A156S 1/1 5-CQA/CoA (sample 1097); and Panel (D) H154N/A155L/A156S 1/10 5-CQA/CoA (sample 1098).

[0064] Fig. 37: HPLC profiles of reaction products generated by the H154A mutant HCTs from 5-CQA and various acyl donors. Reactions were set up with 10 mM 5-CQA and either 0.5 mM CoA/ caffeoyl-/ feruloyl-/ coumaroyl-CoA in 0.1 M sodium phosphate pH 6.5. The reaction was started by adding 0.5 µM enzyme. Samples were analysed after overnight incubation using HPLC-PDA with the acetonitrile method. Panel (A): Control with no enzyme 5-CQA + CoA (sample 1234); Panel (B) 5-CQA + CoA → caffeoyl-CoA + diCQAs (sample 1258); Panel (C): 5-CQA+ caffeoyl-CoA → diCQAs (sample 1259); Panel (D): 5-CQA + feruloyl-CoA → diCQAs + mixed caffeoylferuloylquinic acids (*) (sample 1260); and Panel (E) 5-CQA + coumaroyl-CoA → diCQAs + mixed coumaroylcaffeoylquinic acids (*) (sample 1261). Panels D and E also include an inset enlarging the di-ester HPLC profile.

[0065] Fig. 38: HPLC profiles of the reaction products synthesised by H154A mutant HCTs from 5-FQA and various acyl donors. Reactions were set up with 10 mM 5-FQA and either 0.5 mM CoA/ caffeoyl-/ feruloyl-/ coumaroyl-CoA in 0.1 M sodium phosphate pH 6.5. The reaction was started by adding 0.5 µM enzyme. Samples were analysed after overnight incubation using HPLC-PDA with the acetonitrile method. Panel (A): Control with no enzyme 5-FQA + CoA (sample 1238); Panel (B) Control with no enzyme 5-FQA + coumaroyl-CoA (sample 1241); Panel (C) 5-FQA + CoA → new peaks = diFQAs (putative)(sample 1262); Panel (D) 5-FQA + caffeoyl-CoA → new peaks = caffeoylferuloylquinic acids (putative)(sample 1263); Panel (E) 5-FQA + feruloyl-CoA → new peaks = diFQAs (putative)(sample 1264); and Panel (F) 5-FQA + coumaroyl-CoA → new peaks = coumaroylferuloylquinic acids (putative) (sample 1265).

[0066] Fig. 39: HPLC profiles of the reaction profiles synthesized by 4CL and HCT or HQT from coumaric or caffeic acid and various acyl donors. Reaction mixtures containing 0.5 mM coumaric/ caffeic acid, 0.5/ 10 mM quinic/ shikimic acid, 2.5 mM CoA, 2.5 mM ATP and 5 mM MgCl2 in 0.1 M sodium phosphate pH 6.0 were prepared in a 500 µL final volume. Reactions were started by adding 0.4 µM Nt4CL2 and incubated at 35°C. 75 µL-volume samples were taken at t= 10 min and 5-fold dilutions were analysed using HPLC-PDA with the methanol method. A t= 15 min, 0.5 µM HCT/ HQT was added to the remaining 425 µL reaction mixture. Samples were taken at t= 30 min (15 min after adding HCT/ HQT), t= 105 min and overnight (data not shown for the latter two time points) were also analysed. Panel (A) control with no enzyme and 0.5 mM coumaric acid; Panel (B) 4CL and 0.5mM coumaric acid; Panel (C)

consecutive reactions with 4CL and HQT with coumaric (0.5 mM) and quinic (0.5/ 10 mM) acids; Panel (D) consecutive reactions with 4CL and HCT with coumaric (0.5 mM) and shikimic (0.5/ 10 mM) acids; Panel (E) 4CL and 0.5mM caffeic acid; Panel (F) consecutive reactions with 4CL and HQT with caffeic (0.5 mM) and quinic (0.5/ 10 mM) acids; Panel (G) consecutive reactions with 4CL and HCT with caffeic (0.5 mM) and shikimic (0.5/ 10 mM) acids

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0067] The following abbreviations may be used herein:

4CL	4-coumarate CoA ligase
ACS	acetyl-CoA synthase
AMP	adenosine monophosphate
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
AU	asymmetric unit
BME	β-mercaptoethanol
bp	base pair
C3H	coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol reductase
CAT	chloramphenicol acetyltransferase
CBL	chlorobenzoate CoA-ligase
Cc	*Coffea canephora*
CCR	cinnamoyl-CoA reductase
cDNA	complementary deoxyribonucleic acid
CGA	chlorogenic acids
CoA	coenzyme A
CQA	caffeoylquinic acid
CrAT	carnitine acetyltransferase
CSA	caffeoylshikimic acid
CV	column volume

DA	dactylifric acids
DAHP	3-dehydroxy-d-arabino-heptulose-7 phosphate
DAT	deacetylvindoline 4-O-acetyltransferase
DLS	dynamic light scattering
dNTP	deoxynucleotide triphosphate
DON	deoxynivalenol
DTT	dithiothreitol
DW	dry weight
EMBL:	European Molecular Biology Laboratory
EPSP	5-enolpyruvate shikimate 3-phosphate
EST	expressed sequenced tag
FPLC	fast protein liquid chromatography
FQA	feruloylquinic acid
GST	glutathione S-transferase
HCA	hydroxycinnamic acid
HCBT	hydroxycinnamoyl/benzoyl-CoA anthranilate N-hydroxycinnamoyl/ benzoyltransferase
HCT	hydroxycinnamoyl-CoA shikimate/ quinate hydroxycinnamoyltransferase
HPLC	high-performance liquid chromatography
HQT	hydroxycinnamoyl-CoA quinate/ shikimate hydroxycinnamoyltransferase
HTX	high-throughput crystallography
IPTG	isopropyl β-d-1-thiogalactopyranoside
K-mutant	K210A/K217A mutant (CcHCT)
kDa	kilo Dalton (103 Da)
LB	Luria-Bertani
MaT	malonyltransferase
MES	2-(N-morpholine)-ethane sulphonic acid
MR	molecular replacement
MS	mass spectrometry
MW	molecular weight
MX	macromolecular crystallography

NCBI	National Center for Biotechnology Information
NRPS	non ribosomal peptide synthase
Nt	*Nicotiana tabacum*
NTA	nitriloacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia lyase
PapA5	polyketide-associated protein A5
PCR	polymerase chain reaction
PDB	protein data bank
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PMSF	phenylmethanesulfonyl fluoride
PPi	pyrophosphate
RMSD	root mean square deviation
RT	retention time
SBP	substrate binding pocket
SDS	sodium dodecylsulfate
SM	secondary metabolites
SUMO	small ubiquitin-related modifier
TRI101	trichothecene 3-O-acetyltransferase
UV	ultraviolet
VS	vinorine synthase

[0068] The terms "chlorogenic acid" or "chlorogenic acid species" or "CGA" are used herein essentially synonymously. Chlorogenic acids include all esters of quinic acid (1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid) with cinnamic acid or a cinnamic acid derivative. Common examples of chlorogenic acids include those shown in Table 2. For convenience, the expression "chlorogenic acids and related esters" is sometimes used herein. "Chlorogenic acids and related esters" includes chlorogenic acid species, as well as dactylifric acid species, and other closely related phenylpropanoid compounds found in plants.

[0069] The terms "dactylifric acid" or "dactylifric acid species" or DA are also used synonymously herein. Dactylifric acids is used to refer to esters of shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) with cinnamic acid or a cinnamic acid derivative.

[0070] As used herein "a coffee product" is any product comprising any part of a coffee plant, preferably coffee beans or coffee cherries, and intended for oral consumption by a human or animal. A coffee product may be a pure soluble, or instant, coffee product, which is a dried coffee extract useful for preparing a coffee beverage by dissolution in water.

[0071] The term "food" or "food product" or "food composition" means a product or composition that is intended for ingestion by an animal, including a human, and provides nutrition to the animal.

[0072] As used herein, an amino acid residue can be determined to "correspond" to an amino acid in a related sequence, such as a reference sequence, when the amino acids sequences are first aligned so as to reflect what is known about the conserved motifs, secondary structures, catalytic residues or other features or putative features. Thus, gaps may be present in the alignments to allow for better fit. The skilled artisan will be familiar with various computer models and software packages that provide for aligning related sequences and allow determination of "corresponding" amino acids. Sequence alignments may also be adjusted on the basis of data such as 3-dimensional models, crystallization results, mutagenesis studies and more, either by a computer program, or after visual inspection by a skilled artisan familiar with such alignments.

[0073] The term "microorganism" encompasses at least bacteria, molds and other fungi, and yeasts. A "recombinant microorganism" for purposes herein is one expressing an exogenous gene encoding one or more proteins of interest, particularly an HCT, HQT, or 4CL enzyme.

[0074] As used throughout, ranges are used herein in shorthand, so as to avoid having to set out at length and describe each and every value within the range. Any appropriate value within the range can be selected, where appropriate, as the upper value, lower value, or the terminus of the range.

[0075] As used herein and in the appended claims, the singular form of a word includes the plural, and vice versa, unless the context clearly dictates otherwise. Thus, the references "a", "an", and "the" are generally inclusive of the plurals of the respective terms. For example, reference to "an enzyme", "an amino acid residue", or "an alteration" includes a plurality of such

"enzymes", "residues", or "alterations". Reference herein, for example to "an antioxidant" includes a plurality of such antioxidants, whereas reference to "pieces" includes a single piece. Similarly, the words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. Likewise the terms "include", "including" and "or" should all be construed to be inclusive, unless such a construction is clearly prohibited from the context. Where used herein the term "examples," particularly when followed by a listing of terms is merely exemplary and illustrative, and should not be deemed to be exclusive or comprehensive.

[0076] The methods and compositions and other advances disclosed here are not limited to particular methodology, protocols, and reagents described herein because, as the skilled artisan will appreciate, they may vary. Further, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to, and does not, limit the scope of that which is disclosed or claimed.

[0077] Unless defined otherwise, all technical and scientific terms, terms of art, and acronyms used herein have the meanings commonly understood by one of ordinary skill in the art in the field(s) of the invention, or in the field(s) where the term is used. Although any compositions, methods, articles of manufacture, or other means or materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred compositions, methods, articles of manufacture, or other means or materials are described herein.

[0078] All publications and other references (including patents, published patent applications, publications, texts, and technical and/or scholarly articles) cited or referred to herein are in their entirety incorporated herein by reference to the extent permitted by applicable law. Full citations for publications not cited fully within the specification are set forth at the end of the specification. The discussion of any publication or reference is intended merely to summarize the assertions made therein. No admission is made that any such patents, patent applications, publications or references, or any portion thereof, are relevant, material, or prior art. The right to challenge the accuracy and pertinence of any assertion of such patents, patent applications, publications, and other references as relevant, material, or prior art is specifically reserved.

The Invention

[0079] In one aspect, the invention provides at least one recombinant plant enzyme that is a wild-type HCT, HQT, or 4CL expressed in a microbial cell. The recombinant enzyme is particularly useful for producing commercially useful quantities of one or more chlorogenic acids species or related esters. The recombinant enzyme has enzyme activity substantially identical to a corresponding enzyme isolated from a *Coffea spp.* plant. The enzyme activity may be substantially identical in terms of substrate specificity and preferences, temperature stability and temperatures of activity, pH stability and pH activity profile, products produced, and the like. Presently preferred plants for HCT and HQT are *Coffea canephora* and *Coffea arabica*. For 4CL enzymes, *Nicotiana tabacum* is preferred in addition to *Coffea* spp.

[0080] The recombinant enzymes, singly or in combination with one another, are preferentially expressed in large quantities in *E. coli*, *S. cerevisiae*, or a food-grade lactic acid bacterium, such as a *Lactobacillus*. Food-grade lactic acid bacteria are useful for production of various food components, or for the production of fermented foods. Such organisms may be used to produce chlorogenic and dactylifric acids *in vivo*, for example in large-scale fermentations. They may also be used to produce a supply of enzymes for *in vitro* production of CGAs and DAs.

[0081] In one embodiment, the recombinant enzyme(s) is/are expressed in a probiotic microorganism. Probiotics include many types of bacteria but generally are selected from four genera of bacteria: *Lactobacilllus* (e.g. *L. acidophilus*), *Bifidobacteria*, *Lactococcus*, and *Pediococcus*. Other beneficial species include *Enterococcus* and *Saccharomyces* species. Probiotics are live microorganisms that have a beneficial effect in the prevention and treatment of specific medical conditions when ingested, and may colonize the gastrointestinal tract of an animal. The recombinant lactic acid bacteria or probiotic organism may produce useful or beneficial CGA or DA species, for example, in the gut of an animal consuming such microorganisms. Probiotics generally enhance systemic cellular immune responses and are useful as a dietary supplement to boost natural immunity in otherwise healthy adults. Including the recombinant enzymes HCT, HQT or 4CL in such organisms may provide another opportunity to increase functionality of useful probiotic organisms.

[0082] One or more of the recombinant enzymes can be made in the microorganism in substantial quantities. The protein can be produced in the organism such that it is secreted, for example into an extracellular medium, or it can remain within and accumulate in the cell and obtained by disrupting the cells. The expression of the recombinant enzyme can be constitutive or inducible, depending for example, on the promoter used to drive the expression. In one embodiment, the recombinant enzyme can be partially or substantially purified from the microorganism with a convenient and preferably rapid method, for example using a simple chromatographic separation, affinity chromatography, or the like. The skilled artisan will appreciate that any of a wide variety of methods can be used for such purification steps. In a preferred embodiment, the recombinant enzyme can be purified to near-homogeneity or even to homogeneity as determined by gel filtrations or gel electrophoresis. The skilled artisan will also appreciate that the purification can be aided by including certain features in the expressed gene. In one embodiment, the gene encoding the recombinant enzyme is engineered to provide a purification tag on the N-terminus of the recombinant enzyme. Examples of such tags include hexahistidine ($His_{6)}$ tags which are well-known in the art.

[0083] The recombinant enzyme is readily adapted for *in vitro* production of chlorogenic and/or dactylifric acid species, preferably in commercially-useful quantities. In one embodiment, the enzyme is immobilised on or covalently attached to a matrix that is part of an *in vitro* production system for useful chlorogenic acid species and related esters. For example, CGA or DA compounds may be produced in one or more columns wherein 4CL is used to produce HCA-CoA compounds, and subsequently the HCA group is transferred to acyl acceptor such as quinic or shikimic acid. The production system can be run in a continuous fashion, or in semi-continuous or even batch mode. As another example, the recombinant enzymes, e.g., 4CL, HCT and/or HQT can be used in combination to produce useful chlorogenic acid species and related esters.

[0084] Preferably, the gene encoding the recombinant enzyme is engineered to provide more optimum codon usage for the microorganism in which it is to be expressed. Codon usage tables are available to show preferences for codon usage in various organisms. The skilled artisan will be familiar with such preferences and understand how to adapt a sequence for the microorganism selected to express the recombinant enzyme.

[0085] In various embodiments, the recombinant enzyme is a 4CL enzyme capable of catalysing the production *in vitro* of a hydroxycinnamic acid (HCA)-CoA thioester from an HCA in the presence of coenzyme A (CoA), a divalent metal ion, and adenosine triphosphate (ATP). The recombinant enzyme preferably is catalytically active *in vitro* from at least pH 6.0 to pH 7.5.

[0086] The recombinant 4CL catalyses the production *in vitro* of a CoA thioester from at least cinnamic acid, coumaric acid, caffeic acid, ferulic acid, and sinapic acid, and under suitable condition is capable of converting at least about 80% of the cinnamic acid, coumaric acid, caffeic acid, or ferulic acid to the corresponding HCA-CoA. In one embodiment, an 80% conversion can be accomplished within about 10 to 20 minutes of incubation with recombinant enzyme. In other embodiments, the time for 80% conversion is 30, 40, 50, or 60 minutes. The skilled artisan will appreciate that time required to convert a given amount of substrate can be reduced by increasing the amount of catalyst (enzyme) present in the reaction. The amount of conversion of substrate to product in various embodiments is at least about 50%, 60%, 70%, 80%, 85% or even higher.

[0087] The recombinant enzyme can preferably produce a HCA-CoA thioester that is substantially free from the HCA acid precursor. The absence of the free acid form can improve the use of the HCA-CoA thioester produced as a substrate in a subsequent reaction, for example by an HCT or HQT enzyme. Typical HCA-CoA products for a recombinant 4CL enzyme include coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, and sinapoyl-CoA, depending of course on the HCA substrate used, which can be determined based on the product desired.

[0088] The recombinant enzyme can also be a HCT or HQT enzyme capable of catalysing the production of a chlorogenic acid species or related ester from a HCA-CoA thioester and an acyl acceptor molecule. Preferred HCA-CoA thioesters comprise one or more of the thioesters coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA, depending on the desired CGA or DA to be produced. Presently preferred acyl acceptors comprise quinic acid, shikimic acid, or a mixture thereof. The skilled artisan will understand that while these acyl acceptors are presently preferred and known to perform well, other acyl acceptors that are synthetic or that are found in nature may be useful herein.

[0089] Recombinant HCTs generally have a preference for shikimic acid as the acyl acceptor, whereas recombinant HQTs generally have a preference for quinic acid as the acyl acceptor.

[0090] The recombinant HCT or HQT enzymes are capable of catalysing the production *in vitro* of a HCA-CoA thioester from a chlorogenic acid or related ester, such as a dactylifric acid, in the presence of CoA. The recombinant enzymes preferably demonstrate similar catalytic activity over the temperature range of at least 22-42 °C, i.e. over that temperature range the catalytic activity does not vary in any substantial manner, despite the nearly 20 °C temperature range.

[0091] Surprisingly, the inventors have found that the recombinant enzymes can produce/synthesize *in vitro* one or more diester species of CGA and DA. This is believed to be the first such demonstration of *in vitro* biosynthesis of such compounds. In one embodiment, recombinant HCT can catalyse the production of a HCA-CoA thioester that is a di-, tri- or higher CGA or related ester. Examples of such compounds include 3,4 diCQA, 4,5-diCQA, 3,5-CQA, diferuloylquinic acid species, as well as mixed diesters such as caffeoylferuloylquinic acid species.

[0092] The inventors have also discovered a previously unknown reaction wherein HCT, at least in the presence of CoA, and/or one or more HCA-CoA, can rearrange or "remodel" CGA and DA species to form more complex (di-, tri-, tetraesters) CGAs of DA. This provides an opportunity to generate novel CGA and DA species that could possess heretofore unrecognized properties or benefits.

[0093] Published sequences useful for the amino acid sequence of the recombinant enzyme are available. In various embodiments, the recombinant enzyme has an amino acid sequence of SEQ ID NOs: 23, 44, or 47, or the sequence of any other published sequence of a suitable enzyme from a *Coffea* genome.

[0094] A related aspect of the invention features recombinant microorganisms comprising one or more genes for one or more expressible exogenous enzymes comprising *Coffea* HCT and/or HQT; wherein the microorganism expresses the enzyme(s). The recombinant microorganism is preferably a food-grade lactic acid bacterium.

[0095] In one embodiment, the recombinant microorganism can produce a chlorogenic acid or dactylifric acid species in an amount not naturally produced in a comparable microorganism that does not express the enzyme.

[0096] The microorganism can conveniently be any microorganism, but particularly as described above with respect to the microorganisms, lactic acid bacteria, and probiotic organisms of the prior aspect of the invention.

[0097] In another of its several aspects, the invention provides mutant plant HCT and HQT enzymes. In one embodiment, the mutant HCT or HQT comprises an amino acid sequence that is at least 80% identical to that of a corresponding wild-type HCT or wild-type HQT enzyme found in a plant, but which contains an altered amino acid residue in one or more positions relative to corresponding the wild-type enzyme. In other embodiments, the mutant enzyme may be greater than about 80% identical, for example, at least about 85 or about 90% identical to the wild-type enzyme. In yet other embodiments, the amino acid sequences are at least 91, 92, 93, 94, 95, 96, 97, 98, 99% or 100% identical. Preferably, the mutant can bind at least one substrate or can catalyse the formation of at least one product in either the forward or reverse direction of the reaction transferring an acyl moiety from a CoA thioester to an acceptor molecule to produce a CGA. For example, Figure 16 shows a typical reaction catalysed by the HCT and/or HQT enzymes.

[0098] The mutant enzymes generally have an alteration comprising a substitution, deletion, or addition of one or more amino acid residues. The alteration in some embodiments comprises a conservative substitution of one amino acid residue for a similar or related amino acid, for example a V residue could be conveniently substituted for an L or I residue, or vice versa. The skilled artisan will appreciate the generally-accepted notions of conservative amino acid substitutions and further details are available in standard Biochemistry, Molecular Biology or Protein Engineering texts.

[0099] In various embodiments, the mutant enzymes feature an altered amino acid residue at a position corresponding to an amino acid in a reference amino acid sequence. A preferred reference sequence for mutant HCTs is the *Coffea canephora* HCT amino acid sequence. The amino acid of *C. canephora* HCT is known in the art, and is set forth as SEQ ID NO:23. In accordance with the present invention, the crystal structure of *C. canephora* HCT has been resolved, including co-crystallization with one or more substrates, and the substrate binding pocket(s) and other important features of the enzyme have been determined (data not shown). Based on these data, the inventors have been able to ascertain for the first time the amino acids responsible for binding substrates and for catalysis, as well as those residues closely associated

with those important features. Thus, among presently preferred amino acid positions include those known to be involved with substrate binding, or located close to such residues, as well as residues likely involved with catalysis, or with important conformational features. In various embodiments, the mutant HCT contains an alteration at an amino acid that corresponds to one or more of the following positions in the reference sequence of *C. canephora* HCT (SEQ ID NO:23) (the letter is the one-letter code for the amino acid residue in the reference sequence and the number represents the position of that residue in the reference sequence): W23, N26, V27, D28, L29, V30, V31, P32, N33, F34, H35, T36, P37, S38, V39, Y40, P110, R115, G143, G144, V149, G150, M151, R152, H153, H154, A155, A156, D157, G158, F159, S160, G161, L162, H163, F164, I165, Y203, K210, K217, L231, N248, Y252, Y255, L272, V274, D275, Q276, L280, Y281, I282, A283, T284, D285, R289, L294, N301, V302, I303, F304, T305, L331, L346, K353, L355, V356, R357, G358, A359, H360, T361, F362, K363, C364, P365, N366, L367, G368, I369, T370, S371, W372, V373, R374, L375, P376, I377, G383, M390, G391, P392, G393, G394, I395, A396, Y397, E398, G399, L400, S401, or F402. It is contemplated that combinations of amino acids corresponding to these positions will be the subject of alteration in mutant HCTs.

[00100] A preferred reference sequence for mutant HQTs is a *Coffea canephora* HQT amino acid sequence. The amino acid of *C. canephora* HQT is known in the art, and is set forth as SEQ ID NO:44. The skilled artisan will appreciate the close relationship among sequence for HQTs from different plants, as well as that between HQTs and HCTs from different plants. Based on these data, the inventors have been able to predict the amino acids responsible for docking/binding substrates and for catalysis of the CcHQT, as well as those residues closely associated with those important features.

[00101] In various embodiments, the mutant HQT contains an alteration at an amino acid that corresponds to one or more of the following positions in the reference sequence of *C. canephora* HQT (SEQ ID NO:44) (the letter is the one-letter code for the amino acid residue in the reference sequence and the number represents the position of that residue in the reference sequence): W23, N26, I27, D28, L29, L30, V31, A32, R33, I34, H35, I36, L37, T38, V39, Y40, P110, R115, G143, A144, A149, G150, V151, Q152, H153, N154, L155, S156, D157, G158, V159, S160, S161, L162, H163, F164, I165, Y203, K210, L217, I231, N245, E246, G247, Y252, L272, N274, D275, Q276, L280, Y281, V282, A283, T284, D285, R289, L294,

N301, V302, I303, F304, T305, L331, L346, S353, L355, V356, R357, G358, D359, R360, H361, F362, A363, S364, P365, N366, L367, N368, I369, N370, S371, W372, T373, R374, L375, P376, F377, G383, I390, G391, P392, A393, I394, I395, L396, Y397, E398, G399, T400, V401, or Y402.

[00102] In some embodiments, the mutant HCT or HQT has improved ability to bind at least one substrate or to catalyze the formation of at least one product in either the forward or backward direction, relative to the corresponding wild-type HCT or HQT. In other embodiments, the mutant enzymes feature other properties that make them advantageous relative to the wild-type enzymes on which they are based. For example, the mutant may provide stability over a wider pH, or improved thermostability, or may preferentially catalyse formation of a desired product, or allow a reaction to proceed fully in one direction. In one embodiment, the mutant, preferably a mutant HCT, is useful for producing di- or triesters of CGS or DA species, or even tetraesters of these acids (e.g. quinic or shikimic acids). In yet other embodiments, the mutant is more readily produced as a soluble, active enzyme in a recombinant system, or is more amenable to immobilization on a matrix for use in *in vitro* synthesis systems. In yet other another embodiment, the mutant enzyme is more resistant to proteolysis than the wild-type enzyme, particularly in a recombinant expression system, such as a recombinant microorganism.

[00103] The wild-type HCT or HQT is preferably from a plant that is relatively abundant in chlorogenic acids or dactylifric acid species in nature, including mono-, di-, tri-, tetraesters, or other complex esters. Many monocot and dicots have been found to contain chlorogenic acid and dactylifric acid species and the HCTs and HQTs found in them are contemplated for use herein. Examples of such plants include the *Rubiaceae* (e.g. coffee, plums), *Rosaceae* (e.g. apple, pear, peach), *Solanaceae* (e.g. tobacco, tomato, potato, eggplant), and *Asteraceae* (e.g. artichoke, sunflower, chicory), as well as the model plant *Arabidopsis*. Among presently preferred plant HCTs are those from *Coffea canephora*, *Coffea arabica*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Cynara cardunculus*, *Trifolium pratense*, and *Arabidopsis thaliana*. *Coffea* HCT sequences are particular preferred as wild-type HCT sequence from which to make mutant HCTs because coffee plants tend to among the highest concentrations of CGA species and related compounds, such as DAs, known. *Nicotiana tabacum*, and *Coffea* spp are among the preferred plants for wild-type HQT at present.

[00104] The skilled artisan will appreciate that public databases such as those provided by NCBI and EMBL make amino acid sequences readily available, and are good sources for suitable sequences for HCT and HQT enzymes. Particular HCT amino acid sequences contemplated for use herewith include those with database Accession Numbers ABO47805 (*C. canephora*), CAJ40778 (*C. arabica*), ABO40491 (*C. arabica*), CAD47830 (*N. tabacum*), AAZ80046 (*C. cardunculus*), NP_199704 (*A. thaliana*), ACI28534 (*T. pratense*), and ABO52899 (*P. radiata*). HQT sequences contemplated for use herein include CAE46933 (*S. lycospersicum)*, DQ20063 (*S. tuberosum*), CAE46932 (*N. tabacum*), ABO77956 (*C. canephora*), ABK79690 (*C. cardunculus*), and ABK79689 (*C. scolimus*).

[00105] The skilled artisan will also appreciate that in some cases an amino acid sequence has not been identified, or fully sequenced, and in other cases only a gene sequence encoding a HCT or HQT is available. It is routine to use a deduced amino acid sequence, or to use a partial sequence to obtain a full-length clone for a sequence of interest. Thus obtaining the required information for producing a mutant HCT or HQT enzyme from a large variety of wild-type HCT or HQT sequences is either available or can be obtained using standard methodologies.

[00106] In some embodiments the mutant enzyme may have no particular substrate preference with respect to quinic acid or shikimic acid, for example the enzyme will bind both substrates with similar avidity or affinity and/or will catalyze reactions using either substrate at similar rates, and make similar amounts of product from each substrate. However, the mutant enzymes do show a substrate preference for quinic acid species or shikimic acid species relative to the wild-type enzyme in some embodiments. The enzyme may preferentially bind one substrate over the other, or may catalyze product formation more quickly or more completely with one substrate over the other under similar conditions. In one embodiment, the mutant HCT has a preference for shikimic acid species as a substrate relative to the wild-type HCT. In another embodiment, a mutant HQT prefers quinic over shikimic acid as an acyl acceptor. In other embodiments, the preference may be reversed.

[00107] The mutant enzymes can generally catalyze the formation of mono-, di-, tri, or tetraesters with similar proficiency as the wild-type in which it is based. In various embodiments the mutant HCT in particular provides an improved ability to catalyze to formation

of di-, tri-, and higher esters relative to the wild-type enzyme. In one embodiment, the mutant HCT more proficiently produces diCQA than the wild-type HCT from which it is derived.

[00108] The mutant HCT comprises an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HCT sequence (SEQ ID NO:23) in various embodiments. The mutant may comprise any one or more of the aforementioned alterations, and preferably each alteration is a conservative substitution. The specific mutations H35A, H153A, H154A, A155L, A156S, D157A, Y252A, Y255A, R374E, K210A/K217A, or H154N/A155L/A156S, or combinations thereof may also be useful. The mutant HCT in one embodiment has an amino acid sequence that is any of SEQ ID NOs: 25, 27, 29, 31, 33, 35, 37, 39, or 41.

[00109] In other embodiments, the mutant HQT comprises an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HQT sequence (SEQ ID NO:44). The mutant may comprise any one or more of the aforementioned alterations, and preferably each such alteration is a conservative substitution. The specific mutations 41H35A, H153A, N154A, L155A, S156A, D157A, Y252A, R374E, K210A, or combinations thereof may also be useful. The mutant HQT in one embodiment has an amino acid sequence that is SEQ ID NO:46.

[00110] In yet another aspect of the invention, coffee products containing synthesized chlorogenic acid species and methods for making such coffee products are provided herein. As used herein "a coffee product" may be any product based on any part of a coffee plant, e.g. based on coffee beans or coffee cherries, and intended for oral consumption by a human or animal. A coffee plant may be any *Coffea* species of, preferably *C. arabica* or *C. canephora*. Coffee cherry is the fruit of the coffee plant containing the seeds, called coffee beans. Coffee beans are typically obtained by removal of the fruit pulp from the coffee cherries, e.g. after fermenting the cherries in water or drying the cherries in the sun to facilitate the release of the pulp from the beans. Coffee beans may be used as so-called "green", or raw, coffee beans for production of coffee products, or they may be roasted. Roasting is a heating process that results in a darker colour and formation of the aroma compounds that gives roasted coffee its typical aroma. Coffee beans, green or roasted, may be ground and sold as such for brewing of coffee, e.g. in multi-serve packs for use in conventional home brewers, or in single-serve

capsules for use in dedicated brewing machines. Coffee beans may be decaffeinated by methods known in the art if a decaffeinated coffee product is desired.

[00111] A coffee product may be based on an extract of coffee beans, either whole or ground. Coffee beans to be extracted may be green (raw) coffee beans or roasted coffee beans, or a mixture thereof. Extraction of coffee beans with water and/or steam is well known in the art, e.g. from EP 0916267. The most volatile aroma components may be stripped from the beans before extraction, e.g. if the extract is to be used for the production of pure soluble coffee. Methods for stripping of volatile aroma components are well known in the art, e.g. from EP 1078576. A coffee extract may be sold and used as such, e.g. in the form of a ready-to-drink (RTD) coffee beverage, or it may be concentrated, e.g. by evaporation or membrane filtration, and sold as a liquid concentrate useful as an ingredient in food or beverage products, or for direct use as a coffee beverage with or without dilution. An RTD coffee product may contain additional ingredients, such as e.g. a creamer, milk, milk protein, milk fat, vegetable fat, vegetable protein, sugar, artificial sweetener, stabilizer, and/or other ingredients conventionally used in coffee products. A coffee product may be a pure soluble, or instant, coffee product. A pure soluble coffee product is a dried coffee extract useful for preparing a coffee beverage by dissolution in water. A pure soluble coffee product may be produced by drying a coffee extract obtained as described above. Often the coffee extract will be concentrated, e.g. by evaporation, before drying, e.g. by spray drying or freeze drying. If volatile aroma has been stripped from the coffee beans before extraction, this aroma may be added back to the extract, e.g. before and/or after drying, to improve the aroma of the soluble coffee product. A pure soluble coffee product may be mixed with additional dry ingredients, such as e.g. a creamer, milk, milk protein, milk fat, vegetable fat, vegetable protein, sugar, artificial sweetener, stabilizer, and/or other ingredients conventionally used in coffee products, to produce an instant beverage product.

[00112] In various embodiments, the coffee product additionally comprises one or more exogenous or added chlorogenic acid and/or dactylifric acid species produced or synthesized in accordance with the methods provided herein. Preferably the chlorogenic acid species provides an improved flavour profile, or has been associated with improved flavours in coffee. Examples of such chlorogenic acid species include diCGA species such as 3,4 dicaffeoylquinic acid. Moreover, the exogenous CGA should contain low amounts of

compounds that have been associated with undesirable flavours, for example bitternness or excess astringency, such as 5-feruloylquinic acid (5-FQA).

[00113] In one embodiment, a composition comprising a synthetic chlorogenic acid species is added to a coffee product before the completion of manufacture. Preferably, the chlorogenic acid species is at least partially purified, although in some cases a complex mixture comprising one or more chlorogenic acids synthetically produced, and one or more precursors, substrates, or other products may be present, provided that all such compounds, precursors, or products are food-grade and/or approved for use in foods. In one embodiment, the chlorogenic acid species is synthesized completely *in vitro*, for example in an enzymatic production system, such as an immobilised enzyme system comprising one or more of the enzymes disclosed herein.

[00114] In another embodiment, a recombinant organism that has been modified to contain the one or more of the enzymes disclosed herein is used to at least partially synthesize the chlorogenic acid species or a precursor thereto. A single chlorogenic acid species may be useful in some instances, for example where it contributes to a particularly advantageous flavour profile, or provides a desirable note or other attribute to the product. In other embodiments, a blend of two or more chlorogenic acid species may be more useful. In such cases, the chlorogenic acid and related species may be synthesized separately and subsequently blended, or they by co-synthesized in an in vitro system, or in a recombinant organism adapted for such synthesis by the inclusion of one or more of the enzymes disclosed herein.

[00115] Preferably, the chlorogenic acid species included with the coffee product are products that are not readily available from natural sources and which cannot be readily (or affordably) synthesized by other means in commercially useful quantities. In other embodiments, the chlorogenic acid species have been associated with one or more beneficial effects on human health. The chlorogenic acid may be monomeric chlorogenic acid species, or di-, tri- or more complex chlorogenic acid species. Examples of preferred chlorogenic acid and dactylifric species include caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs), caffeoylshikimic acids (CSAs), coumaroylshikimic acids, feruloylshikimic acids (FSAs), dicaffeoylquinic acids (diCQAs), or other di-, tri- or tetraesters, or combinations thereof.

[00116] In a related aspect, the invention also provides a food product comprising one or more synthetic chlorogenic acid species made in accordance with the methods disclosed herein. The chlorogenic acid species may be added to a product that benefits from the addition of

one or more chlorogenic acid species. In one embodiment, the food product may be a coffee-flavoured product, even if the product contains no coffee *per se*. In another embodiment, the chlorogenic acid species may help to form the flavour profile for a simulated coffee flavouring or other artificial flavouring. In another embodiment, the chlorogenic acid species may be added as a functional food component, for example as an antioxidant, or as an aid to preventing, reversing, or minimizing health problems, such as those associated with cardiovascular disease or cancer.

[00117] Preferably the food product comprising exogenous one or more chlorogenic acid or dactylifric acid species. In one embodiment, the chlorogenic acid or dactylifric acid species are produced *in vitro*.

[00118] The food product is a coffee product as described above in certain embodiments. Presently preferred are soluble coffee product wherein the exogenous chlorogenic acid or dactylifric acid species provide a functional ingredient that improves quality or provides a health benefit, or directly or indirectly provide a desirable flavour attribute, aroma attribute, or colour attribute.

[00119] The exogenous chlorogenic acid or dactylifric acid species are added to the food product as a mixture to the food product during processing in one embodiment. In other embodiments, individual CGA or DA species are added to the food product. Exogenous chlorogenic acid or dactylifric acid species contribute antioxidant properties useful to the food and/or to the person consuming the food in some embodiments. Mono- and diester CGA or CSA species are useful herein, particularly where those compounds have been synthesized or produced in accordance with this disclosure. 3,4, caffeoylquinic acid can be added to a food product at levels that improve the flavour and aroma of the food product as determined by sensory evaluation. In most cases, to avoid low acceptability by a consumer, the CGA and or DA added are substantially free of 5-feruloylquinic acid, although in some foods and beverages additional bitterness or astringency may be desirable.

[00120] In another aspect of the invention compositions are provided comprising one or more recombinant enzymes (as described above), one or more mutant HCTs or mutant HQTs (as described above). The compositions are adapted for *in vitro* synthesis of one or more chlorogenic acid species or dactylifric acid species. The compositions may be provided as kits providing two or more separate packages such that the final composition is mixed by the end-user in order to use the compositions.

[00121] The compositions comprise substrates and cofactors sufficient for the production of one or more chlorogenic acid species or dactylifric acid species. For example, the substrates may comprise coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, sinapoyl-CoA, or combinations thereof, and an acyl acceptor. The cofactors comprise a catalytically useful divalent cation, preferably Mg^{++}, ATP, and optionally, CoA.

[00122] The acyl acceptor generally comprises quinic or shikimic acid species, or a combination thereof. Other acyl acceptors may be used as well.

[00123] In a presently preferred embodiment, one or more of the enzymes is immobilised on or covalently bound to a matrix. Such immobilised enzymes can conveniently be included with a kit for assembly of the final composition by the end user.

[00124] The chlorogenic acid species or dactylifric acid species that can be produced by the composition include caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs), caffeoylshikimic acida (CSAa), coumaroylshikimic acids, feruloylshikimic acids (FSAs), dicaffeoylquinic acids (diCQAs), or other di-, tri- or tetraesters, or combinations thereof. The di-, tri-, and higher esters may be mixed in having different HCAs attached to a single acyl acceptor.

[00125] The composition can also be adapted for *in vitro* production of a HCA-CoA thioester catalysed by a 4CL enzyme, prior to *in vitro* production of the chlorogenic acid species or dactylifric acid species. In this manner, one of the substrates can be produced in the enzyme composition to provide further ease of use for making compounds that are otherwise difficult or impossible to synthesize. The substrates for production of the HCA-CoA thioester generally comprise one or more of cinnamic acid, coumaric acid, caffeic acid, or ferulic acid, and CoA

[00126] In yet another of its several aspects, the invention provides methods of producing a chlorogenic acid or dactylifric acid species *in vitro*.

[00127] The methods comprise the steps of: a) providing an HCT or HQT or both; b) providing substrates and cofactors sufficient to form a chlorogenic acid or dactylifric acid species in the presence of an HCT or HQT or both; and c) contacting the substrates and cofactors with the HCT or HQT, or both. The contacting step is done under conditions permitting enzymatic activity. The enzyme and substrate are maintained in contact under reaction conditions maintained for enough time to form CGA or DA species product(s). he product

comprises a mono-, di-, tri- or tetraester species of chlorogenic or dactylifric acid. The product can be a mono-, di-, tri- or tetraester species of chlorogenic or dactylifric acid. *Coffea* HCTs are particularly useful herein.

[00128] Preferred substrates include at least one hydroxycinnamoyl-CoA thioester, such as coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA. Substrates also include an acyl acceptor, such as quinic acid, shikimic acid, or a combination thereof.

[00129] The chlorogenic acid or dactylifric species preferred as products include caffeoylquinic acids (CQAs), coumaroylquinic acids, feruloylquinic acids (FQAs), caffeoylshikimic acida (CSAa), coumaroylshikimic acids, feruloylshikimic acids (FSAs), dicaffeoylquinic acids (diCQAs), or other di-, tri- or tetraesters, or combinations, as discussed above.

[00130] In one embodiment, the method includes an additional step wherein a HCA-CoA thioester is produced using a 4CL enzyme.

[00131] The method can also include additional substrates such as CoA, and one or more chlorogenic acid or dactylifric acid species. As discussed above, HCT can remodel CGAs or DAs in the presence of HCA-CoA and/or CoA to form further CGAs and/or DAs.

[00132] As with other aspects of the invention involving enzymes, at least one of the HCT, HQT, or 4CL are preferably immobilised on, or covalently bound to, a matrix. The matrix can be any matrix used for enzyme immobilisation, such as a polymer with desirable properties, for example a complex polysaccharide or dextrin, a silica resin, a glass bead, and the like. The skilled artisan is familiar with such matrices and will appreciate how to employ them, and further understand such an immobilisation is only useful where the enzyme retains a significant portion of its activity.

[00133] In a further aspect of the invention, methods of producing a chlorogenic acid or dactylifric acid species *in vitro* are provided. The methods disclosed in this aspect of the invention comprise the steps of: a) providing an HCT, and optionally, an HQT or 4CL or both; b) providing one or more HCA thioesters, an acyl acceptor, CoA, and a first chlorogenic acid or dactylifric acid species; c) contacting the one or more HCA thioesters, acyl acceptor, CoA, and first chlorogenic acid or dactylifric acid species with the HCT under conditions permitting enzymatic activity, and for a time sufficient to permit the formation of product that is a second

chlorogenic acid or dactylifric acid species. The method provides that the first chlorogenic acid or dactylifric acid species is catalytically converted into a second chlorogenic acid or dactylifric acid species by the action of the HCT, or the HCT in combination with an HQT and/or 4CL enzyme. The HCT enzyme is preferably a *Coffea* HCT, particularly a *C. canephora* HCT, and may comprise SEQ ID NO:23, for example. The HQT enzyme is preferably a *Coffea* HQT, particularly a *C. canephora* HQT, and may comprise SEQ ID NO:44, for example. The 4CL enzyme can be a 4CL from tobacco (*Nicotiana tabacum*), examples of which are known in the art. The 4CL enzyme can also be a *Coffea* 4CL, particularly a *C. canephora* 4CL2, and may comprise SEQ ID NO:47.

[00134] Another aspect of the invention features methods of modifying the lignin content and profile in plants. It is expected that reducing lignin in plant material will make this material more useful for many applications, including in biofuel production and plant feed because the biomass will be easier to solubilize and to degrade. By way of background, it has been shown previously that lignin levels can be reduced in plants by decreasing the expression of genes encoding proteins involved in making lignin precursors, particularly the phenylpropanoid pathway enzymes 4CL, HCT and HQT. However, improvements in forage digestibility or saccharification efficiency were offset by poor growth and reduced biomass production. Thus, studies to date have led to the conclusion that reducing lignin via a simple "knock down" of one or more genes of the phenylpropanoid pathway will not generate plants that have both reduced levels of lignin (with better utility for animal fodder and easier enzymatic degradation for biofuels etc) and normal biomass production.

[00135] However, the use of enzymes that favour the production of mono-, di-, and/or triesters of chlorogenic acid and the related shikimate containing compounds opens the opportunity to create plants that have smaller reductions of lignin but identical, or better overall biomass productivity, thereby improving the utility of the plants for important biomass related applications such as use for biofuels or animal fodder. In accordance with the present invention, this can be accomplished by expressing the coffee HCT or HQT enzymes, or the mutants of HCT in the cells of plants in order to shunt some of the phenylpropanoid precursors into mono, di, and tri CGA and related shikimate containing compounds. In addition to having slightly lower levels of lignin, and/or less complex lignin due to the interference of the stored CGA type compounds in lignin formation, it is expected that plant materials with higher CGA will have increased levels

of relatively easily extractable "phenolic related matter" (i.e., the increased levels of soluble CGA). Additional benefits that may be associated with plants with increased CGA and related compounds include increased protection of these plants from oxidative stress-related damage, as well as protection from pathogens such as bacteria and insects.

[00136] The coffee HCT, HQT and mutants thereof described herein can be over-expressed in plants in accordance with methods known in the art, for instance as described in US Patent Pub. No. 20090158456 (describing the use of *Coffea* HCT and HQT genes in the engineering of plants). Transformation protocols may utilize constitutive promoters such as the 35S promoter, or other green tissue specific strong promoters, such as the coffee rbcs promoter described in U.S. Patent No. 7,153,953. Alternatively, tissue specific promoters can be used to increase the CGA production in specific parts of the plant (in tubers for example, or in seeds using seed specific promoters such as the oleosin promoter described in U.S. Patent Publication No. 20090229007. It is also advantageous, for example in crops for forage, to express higher levels of coffee HCT, HQT, or related mutants either late in leaf maturation, or only in the mature, early senescing leaves. One promoter for these late stages of leaf maturation is the coffee cysteine proteinase inhibitor 4 promoter.

[00137] As it is clear that the high levels of chlorogenic acids in the coffee grain are also driven in part by the high level of quinic acid found early during grain maturation, a further improvement in CGA accumulation and lignin amount/structure modifications in plants can be achieved by additionally engineering the plants with coffee HCT/HQT and related mutants, with genes that lead to elevated levels of quinic acid (or shikimic acid). Alternatively, plants to be first used for reduced lignin and increased CGA and related shikimate compounds would be species that have relatively high levels of quinic acid or shikimate in the tissues where the coffee HCT/HQT and coffee HCT mutants are designed to be expressed.

[00138] These and other aspects of the invention will be further understood by reference to the Examples that follow.

EXAMPLES

[00139] The invention can be further illustrated by the following examples, although it will be understood that the examples are included merely for purposes of illustration and are not intended to limit the scope of the invention unless otherwise specifically indicated.

I. Cloning, Expression, and Purification

Introduction

[00140] A protein difficult to isolate in adequate quantities from its natural source can often be produced in sufficient quantities using recombinant expression in bacterial, yeast, insect or mammalian cells (Baneyx, 1999; Kost *et al.*, 1999; Malissard *et al.*, 1999; Hunt, 2005; Baldi *et al.*, 2007). Expression in *E. coli* is generally the easiest, quickest, and cheapest method currently available, however, many eukaryotic proteins do not fold properly in prokaryotic systems, instead forming insoluble aggregates or inclusion bodies. In some cases, the solubility of such proteins can be improved by: (a) decreasing expression temperature; (b) using a fusion partner/ polypeptide tag (Esposito *et al.*, 2006); or (c) refolding (Li *et al.*, 2004). The use of cleavable polypeptide/ protein tags permits simplified affinity purification procedures to obtain sufficient amounts of protein for subsequent characterisation.

Preliminary Cloning

[00141] Cornell University and Nestlé S.A. released 47,000 expressed sequence tag (EST) sequences from *Coffea canephora* (Lin *et al.*, 2005). The EST databank (http://www.sgn.cornell.edu) was used for a BLAST search (Basic Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/) against *Nicotiana tabacum hct* (AJ507825) and *hqt* (AJ582651) gene sequences (Hoffmann *et al.*, 2003; Hoffmann *et al.*, 2004). This search resulted in two hits: the unigenes CGN-U123197 and CGN-U125212, corresponding to the partial open reading frames (ORF) for putative *Coffea canephora* hydroxycinnamoyltransferases. The full length cDNAs were isolated by 5'-RACE-PCR for rapid amplification of cDNA ends followed by the cloning of *Cchct* and *Cchqt* genes (Lepelley *et al.*, 2007). A multiple sequence alignment was generated with ClustalW (Larkin *et al.*, 2007) using acyltransferase nucleotide sequences from different plant species. Sequence comparisons confirmed that one of the cloned sequences lies on a branch containing *hqt* gene sequences, the other in a branch clustering *hct* gene

sequences from different plant species (not shown). *Cchct* and *Cchqt* exhibit 66 % sequence identity at the nucleotide level. The translated sequences contain both the HX3D and DFGWG motifs, which are characteristic of BAHD acyltransferase amino acid sequences.

Production of recombinant 4CL2:

Coffea canephora 4-coumarate CoA ligase isoform 2

[00142] A plasmid provided by Nestlé contained the *Cc4cl2* gene (1626 bp) inserted into the inverted pET28a(+) vector with BamHI (5') and HindIII (3') restriction sites. This plasmid allows for expression of *Cc*4CL2 with an N-terminal His_6-tag. BL21 (DE3) cells were transformed with the plasmid for standard expression tests at 37 and 20 °C. A band corresponding to the expected size of His_6-*Cc*4CL2 with a 24 amino acid linker (62.6 kDa) was detected in the cell extracts using SDS-PAGE and western-blot analysis against the polyhistidine tag (Fig. 2).

[00143] Expression was scaled-up in flasks containing 1 L of Luria-Bertani (LB) medium supplemented with 30 µg/mL kanamycin, which were inoculated with a 1/100 (v/v) overnight starter culture. Cells were grown at 37 °C until the optical density (OD) at λ= 600 nm reached 0.6. After cooling to room temperature, over-expression was induced with 1 mM IPTG and cells were grown overnight at 20 °C. The cells were harvested by centrifugation at 5,000 x g and resuspended in a solution composed of 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 2.5 mM $MgCl_2$, 10 % glycerol and 5 mM β-mercaptoethanol (BME) (buffer A). The cells then were flash-frozen and stored at -80 °C until purification. EDTA-free protease inhibitors tablets, lysozyme and DNaseI were added prior to cell lysis using a French press (2 cycles at 10 kPSI). The lysate was centrifuged for 30 min at 50,000 x g and 4 °C and the supernatant loaded on to a 5 mL HisTrap column (GE Healthcare) using an Äkta Prime system. After washing the column with 10 CV of buffer A and 5 CV of 5 % buffer B (buffer A supplemented with 500 mM imidazole), an elution gradient of 5-100 % buffer B was applied. 1 mL fractions were collected and analysed on 12 % SDS-PAGE gel stained with Coomassie blue. A SDS-PAGE band corresponding to the size of His_6-*Cc*4CL2 was observed in the elution fractions collected from the affinity column (Fig. 2A). Over-expression of the recombinant protein was confirmed by Western-blot analysis against the hexahistidine tag. The fractions containing the protein were pooled and dialysed against 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2.5 mM $MgCl_2$ and 1 mM

DTT. A tag cleavage test was carried out with thrombin protease (Sigma) after dialysing the protein into a buffer composed of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 % glycerol, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 5 mM BME. Cleavage was successful at both room temperature and at 4 °C, but some precipitation occurred. The cleaved *Cc*4CL2 was recovered with subtractive affinity chromatography. Dynamic Light Scattering (DLS) analysis performed at 20 °C indicated that coffee 4CL2 with or without the His_6-tag was highly polydisperse. The over-expression procedure was repeated with Rosetta 2 (DE3) cells (Novagen) to overcome the codon bias for expression in *E. coli*. However, the resulting protein was still aggregated.

Nicotiana tobacco 4-coumarate CoA ligase isoform 2

[00144] Because *Cc*4CL2 required more exploratory work to obtain a fully soluble, monodisperse protein, a synthetic gene encoding *Nicotiana tabacum* 4CL2 (GenBank accession number: U50846) was purchased from Geneart (Regensburg, Germany). This protein had previously been used for the enzymatic synthesis of various hydroxycinnamoyl-CoA thioesters (Beuerle *et al.*, 2002). The codon-optimised gene (1653 bp) was subcloned into the pET21d vector (Novagen) digested with BamHI (5') and XhoI (3') for expression with an uncleavable C-terminal His_6-tag and a two-amino acid (Lys-Glu) linker (60.5 kDa).

[00145] Several *E. coli* strains, namely BL21 (DE3), BL21* (DE3), BL21 (DE3) pLysS and BL21* (DE3) pLysS, were transformed with the pET21d_*Nt4cl2* plasmid for small-scale expression tests. The soluble extracts were analysed by SDS-PAGE (Fig. 3). The highest soluble expression level was obtained with BL21* (DE3) cells, which were subsequently used for large scale production and purification of the recombinant *Nt*4CL2-His_6. Four (4) L of LB medium containing 100 mg/L ampicillin were inoculated with a 1/100 (v/v) overnight starter culture and grown at 37 °C until the OD at λ= 600 nm reached 0.6. The temperature was lowered to 20 °C before inducing the expression with 1 mM IPTG, and then the cells were incubated overnight. After centrifugation for 15 min at 5,000 x g, the supernatant was discarded and the bacterial pellet resuspended on ice in a lysis buffer composed of 50 mM bis-Tris pH 7.0, 10 % glycerol and 10 mM NaCl according to the previously described procedure (Beuerle *et al.*, 2002). In contrast with the authors however, a later comparison with a standard buffering agent (50 mM Tris-HCl pH 8.0) did not show any difference in the protein stability, so this standard buffer was subsequently used.

[00146] Cell lysis was achieved using one cycle at 2.0 kPSI in the One Shot cell disrupter (Constant Systems). The lysate was centrifuged for 30 min at 50,000 x g and 4 °C. The supernatant was then loaded on a 2 mL nickel-nitriloacetic acid (NTA) gravity column (Qiagen). After washing with 10 CV of lysis buffer (see above) and 10 CV of 20 mM Tris-HCl pH 8.0 and 500 mM NaCl, the protein was eluted with 20 mM Tris-HCl pH 8.0, 500 mM NaCl and 500 mM imidazole. The fractions containing the target protein were pooled and dialysed overnight against 20 mM Tris-HCl pH 8.0 and 50 mM NaCl. The protein was further purified by anion-exchange chromatography (MonoQ GL 5/50, GE Healthcare) using a gradient from 50 mM to 1 M NaCl on a Äkta Explorer system at 4 °C. The fractions containing the recombinant protein were pooled, concentrated and loaded on a HiLoad 16/60 Superdex 200 (GE Healthcare) for size-exclusion chromatography in 20 mM Tris-HCl pH 7.5 and 50 mM NaCl (Fig. 4A). The purest fractions from the size-exclusion chromatography were pooled and concentrated to 15 mg/mL in 10 mM Tris-HCl pH 7.5 and 50 mM NaCl (Fig. 4B). The protein was stable in a limited proteolysis experiment on ice with trypsin 1/1000 (w/w) for at least 1 h. Aliquots were flash-frozen and stored at -80 °C for biochemical assays. The remaining protein solution was incubated with 2 mM ATP, 2 mM $MgCl_2$, 1 mM coumaric acid and 1 mM CoA for subsequent crystallisation studies.

Production of recombinant HCT and HQT

HCT and HQT GST-fusion as a starting material

[00147] The cDNAs encoding *Coffea canephora* HCT and HQT (GenBank accession numbers: EF137954 and EF153931 respectively) were provided by Nestlé R&D centre in Tours. They were inserted in the pGTPc103a vector (GTP Technologies), which produces a recombinant protein in fusion with N-terminal glutathione S-transferase (GST). BL21 (DE3) cells were transformed with pGTPc103a_*Cchct* and pGTPc103a_*Cchqt* plasmids and plated on LB-agar medium containing 30 µg/mL kanamycin. Cultures of 100 mL LB medium were grown until the OD at λ= 600 nm reached 0.6 and expression was induced with 1 mM IPTG. Cells were grown for 3 h at 37 °C or overnight at 20 °C. No band corresponding to the size of the full-length GST-HCT and GST-HQT proteins (75.7 and 75.3 kDa respectively) could be detected on a 12 % SDS-PAGE gel stained with Coomassie blue, where the non-induced and induced cell fractions were loaded.

[00148] Like most eukaryotic genes, coffee *hct* and *hqt* display an unfavourable codon distribution in *E. coli* that can limit expression yields (Kane, 1995). Consequently, expression in a strain that co-expresses the transfer RNAs for these rare codons was considered. Rosetta 2(DE3) cells (Novagen) were transformed with each plasmid and plated on LB-agar medium containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol. The full-length GST-HCT and GST-HQT proteins could not be detected on SDS-PAGE, while only a faint band was detected on western-blot together with N-terminal fragments of 25-30 kDa. An affinity chromatography purification step was carried out for GST-HQT over-expressed in Rosetta 2(DE3) at 37 °C for 3 h. The bacterial pellet was resuspended in phosphate buffered saline (PBS) buffer pH 7.4 containing 1 mM DTT. The cells were subjected to three cycles of freezing in liquid nitrogen and thawing in a 25 °C water-bath. The cell lysate was centrifuged for 30 min at 50,000 x g and 4 °C. The supernatant was loaded onto a 5 mL Glutathione Sepharose 4 Fast Flow column (GE Healthcare) equilibrated with PBS buffer pH 7.4. After washing with 5 CV of buffer, the protein was eluted in 50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione and 1 mM DTT. The fractions were analysed by SDS-PAGE with Coomassie blue staining. The elution fractions contained several bands, among which was a band at 75 kDa, corresponding to the expected size of GST-HQT. Western-blot analysis confirmed the production of GST-HQT by detecting the 75 kDa polypeptide, as well as a band at 60 kDa and smeared bands around 25-30 kDa, probably corresponding to truncated N-terminal fragments including the GST fusion (Fig. 5).

HCT and HQT sub-cloning for expression with a hexahistidine-tag

[00149] To improve the expression levels of the full-length proteins, both *Cchct* and *Cchqt* genes were sub-cloned into the pPROEX_HTb vector (Life Technologies) for expression with a cleavable hexahistidine (His_6)-tag at the N-terminus. The target nucleotides were amplified by polymerase chain reaction (PCR) using specifically designed oligonucleotide primers flanked with the appropriate cloning sites (Table 4). The reaction mixtures were made in a final volume of 50 µL as follows: 1 µL of plasmid DNA template, 5 µL of PCR buffer (10x), 1 µL of forward and reverse primers (10 pmol/µL), 200 µL dNTP mix (10 mM) and 0.3 µL (2.5 u) of High Fidelity PCR enzyme mix (Fermentas) or *Pfu* DNA polymerase (Stratagene). A standard PCR program was run with an annealing temperature of 55 °C and an elongation time of 4 min.

The PCR products were analysed using 1 % agarose gel electrophoresis stained with SYBR Safe (Fig. 6). The PCR products, obtained with *Pfu* DNA polymerase, were double digested with the appropriate restriction enzymes and purified by gel extraction (Qiaquick kit, Qiagen). The DNA fragments were ligated into the linearised and dephosphorylated pPROEX_HTb vector. TOP10 cells (Invitrogen) were transformed for plasmid DNA preparation and the insert sequence was verified by DNA sequencing (Genecore, Germany).

Table 4: Oligonucleotide primers used to amplify Cchct and Cchqt

Amplicon	*'chct*
Template	GTPc103a_*Cchct*
Primer 5'	agcactagggatccatgaaaatcgaggtgaaggaatcg (BamHI) (SEQ ID NO:1)
Primer 3'	cgtcgatcactcgagtcaaatgtcatacaagaaactctggaa (XhoI) (SEQ ID NO:2)
Amplicon	*'chqt*
Template	GTPc103a_*Cchqt*
Primer 5'	agcactagggatccatgaagataaccgtgaaggaaaca (BamHI) (SEQ ID NO:3)
Primer 3'	cgtcgatcatctagatcagaaatcgtacaggaacctttg (XbaI) (SEQ ID NO:4)

Expression and buffer tests for hexahistidine-tagged HCT

[00150] Several *E. coli* strains were transformed with the new pPROEX_HTb_*hct* construct and small-scale expression tests performed at 37 °C and 20 °C. The bacterial pellets were resuspended in BugBuster protein extraction reagent (Novagen) for chemical lysis. The soluble cell extracts were loaded onto single-use His-Trap Spin columns (GE Healthcare) containing 100 µL Ni-NTA agarose beads for affinity purification. The fractions were eluted with buffer containing 500 mM imidazole and analysed by SDS-PAGE and western-blot against the His_6-tag. HCT expression levels were highest in BL21* (DE3) pLysS cells grown overnight at 20 °C. Two truncated N-terminal fragments (~25-30 kDa) remained in the insoluble fraction of the cell lysate (Fig. 7).

[00151] To optimise the purification conditions for HCT, 100 mL LB cultures of BL21* (DE3) pLysS were prepared. Expression was induced when the OD reached 0.6/0.9 with 0.5/1 mM IPTG and cells incubated overnight at 20 °C. The pellets were resuspended in different lysis buffers (50 mM Tris-HCl pH 7.4, 300/500 mM NaCl, 20 mM imidazole, 2 mM BME, 0/10

% glycerol, 0/1 % Tween 20) supplemented with lysozyme and DNaseI. The cells were lysed with 4 cycles of freeze and thaw using liquid nitrogen and a water-bath at 25 °C. A similar purification step using His-Trap Spin columns was then performed. No major difference was detected for induction at 0.5 or 1 mM IPTG and at OD at λ= 600 nm of 0.6 or 0.9. The best buffer composition was 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 20 mM imidazole, 2 mM BME and 10 % glycerol.

HCT codon-optimised genes for expression in E. coli

[00152] A synthetic cDNA with improved codon usage was later obtained from Geneart (Regensburg, Germany) to allow a high and stable expression rate of the heterologous protein. The optimised genes for *Cchct* and *Cchqt* were cloned in the pPROEX_HTb expression vector. Similar protein expression levels were obtained with the wild-type and the codon-optimised *hct* genes. The synthetic gene was used in all the following experiments.

Large scale expression and purification of HCT

[00153] The synthetic pPROEX_HTb_*hct* construct was used to over-express *Cc*HCT with an N-terminal His_6-tag in BL21* (DE3) pLysS strain of *E. coli*. A single isolated bacterial colony grown on a freshly streaked plate of LB-agar medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol was used to inoculate a starter culture for overnight growth at 37 °C. Cultures of 1 L LB medium were then inoculated with a 1/100 (v/v) starter culture and incubated at 37 °C until the OD at λ= 600 nm reached 0.6. The flasks were cooled in ice-water and 1 mM IPTG was added for overnight expression at 20 °C. The cultures were centrifuged for 20 min at 5,000 x g and 4 °C and the bacterial pellet produced from a 1 L culture was resuspended in 25 mL buffer composed of 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 % glycerol, 20 mM imidazole and 5 mM BME (buffer A). The cell stock was flash-frozen and stored at -80 °C.

[00154] All subsequent operations were carried out at 4 °C. Prior to cell lysis, lysozyme (~200 µg/mL), DNaseI (~20 µg/mL) and EDTA-free Complete Protease Inhibitor Cocktail Tablets (Roche) were added to the thawing cells and stirred for 15 min. The resuspended cells were ruptured by passage through a French pressure cell (2 cycles at 10,000 PSI). The lysate was then centrifuged at 50,000 x g for 30 min to remove the cell debris. A 5 mL

His-Trap HP column (GE Healthcare) was equilibrated with 10 % (v/v) buffer B (buffer A supplemented with 500 mM imidazole). The soluble fraction of the cell lysate was loaded and a gradient from 10 to 100 % (v/v) buffer B imidazole was applied. The His_6-tagged protein eluted with 250 mM imidazole. The fractions containing the recombinant protein were pooled, incubated with 1/100 (w/w) Tobacco Etch Virus (TEV) protease, and dialysed overnight in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 % glycerol, 1 mM DTT and 0.5 mM EDTA. The TEV cleavage leaves only three residues (Gly-Ala-Met) attached to the target protein. Cleaved HCT was recovered by subtractive chromatography using the same nickel column. The protein sample was concentrated to 500 µL and injected on a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) with 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM BME. HCT eluted at 14.5 mL, which corresponds to the expected size for a monomer of 50 kDa, according to the column calibration curve. The peak fractions were collected and analysed on a 12 % SDS-PAGE with Coomassie blue staining (Fig. 8). Only the purest fractions were pooled, concentrated by centrifugation and flash-frozen at -80 °C. A mean yield of 4.5 mg/L culture was obtained.

Degradation and limited proteolysis of HCT

[00155] A comparison of protein samples produced from batch to batch and stored at different temperatures (-80, -20 and 4 °C) was carried out. It was observed that the purified protein sample usually contained two additional bands around 25-30 kDa that were amplified with time (Fig. 9A). This indicates that the native enzyme is partially degraded into two major fragments by an endogenous endopeptidase activity, probably originating from the crude *E. coli* extract. Size-exclusion chromatography of the degraded sample showed only one symmetric peak of elution corresponding to a full-length protein size (data not shown). This indicates that the fragments remained physically associated throughout the purification procedure. N-terminal sequencing of *Cc*HCT fragments after storage resulted with a limited confidence level in the sequence DSVPE, which maps on Ser218.

[00156] The degradation pattern was reproduced by incubating freshly purified His_6-*Cc*HCT with several proteases. Reaction samples were taken at several time points and supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) to stop the reaction. 12 % SDS-PAGE and western-blot (anti-His_5) analysis revealed two bands of approximately 25 kDa appearing after a few minutes incubation with trypsin, thermolysin or subtilisin. The band of the

full-length protein was barely visible after 10 min incubation (Fig. 9B). The protein was intact in the reactant mixture where no protease was added. The fragment bands were extracted from the polyacrylamide gel and subjected to N-terminal sequencing. This resulted in the identification of the sequence SDSVPETA, which maps after Lys210 that is predicted to be part of the inter-domain loop region.

Chemical modification of CcHCT by reductive methylation

[00157] The reductive methylation of solvent-accessible lysine residues is a simple and inexpensive method to aid in the crystallisation of some proteins. The protocol used was adapted from (Walter *et al.*, 2006) and applied to *Cc*HCT, which contains 20 potential methylation sites (19 Lys and the N-terminal amino group). The reaction was performed at 4 °C with 30 mg of pure protein at 0.65 mg/mL in 50 mM sodium HEPES pH 7.5, 250 mM NaCl and 5 mM BME. 20 µL of freshly prepared 1 M dimethylamine-borane complex (ABC) (Fluka) and 40 µL of 1 M formaldehyde per mg of protein were added to the protein sample and incubated for 1 h. The procedure was repeated once before leaving overnight under gentle agitation. The precipitated material formed during the methylation reaction was removed prior to concentration of the remaining protein sample (76 %). The buffer was exchanged to 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 5 mM BME for size-exclusion chromatography using a Superdex 200.

[00158] Limited proteolysis was then carried out on the native and methylated HCTs. The proteins were incubated at 30 °C with trypsin at a 1/100 (w/w) ratio in a buffer composed of 20 mM Tris-HCl pH 7.5. The reaction was monitored by taking aliquots from t= 0 to 120 min and stopped by the addition of 1 mM PMSF prior to loading the samples on a 12 % SDS-PAGE gel. As expected, the results showed that, contrary to native *Cc*HCT, the methylated derivative was resistant to trypsin proteolysis (Fig. 9C). From these observations and the N-terminal sequencing results (above), it was postulated that the solvent-accessible Lys residues part of the predicted inter-domain loop could have an influence on the stability of *Cc*HCT. Once cleaved, the loose parts may hinder the crystal formation. The two charged amino acids, Lys210 and Lys217, which are characterised by a high conformational energy, were consequently mutated to Ala to engineer a trypsin-resistant protein.

K210A/K217A (K) mutant CcHCT

[00159] K-mutant *Cc*HCT was produced using expression and purification procedures similar to that for the native protein (above). Limited proteolysis with trypsin and chymotrypsin had no or little effect on the K-mutant HCT, while subtilisin could still cut the protein into two stable fragments (Fig. 10).

His_6-HQT expression

[00160] A similar expression protocol was applied to pPROEX_HTb_*Cchqt*. No band corresponding to the correct size of the His_6-*Cc*HQT could be detected in the elution fractions loaded on polyacrylamide gel. However, multiple bands at lower molecular weight (25-30 kDa) were observed in the insoluble and soluble fractions, similar to the expression pattern for GST-HQT (Fig. 5). These bands probably correspond to N-terminal truncated fragments of *Cc*HQT. No further work was carried out with this expression vector as no satisfactory expression conditions were found. A synthetic *Cchqt* gene (Geneart) was also tested, but did not result in any improvement in the expression level of the full-length *Cc*HQT.

Cchqt subcloning in pET-28M-SUMO3

[00161] As *Cc*HQT was difficult to over-express with previous systems, the synthetic *Cchqt* gene was sub-cloned into pET-28M-SUMO3 expression vector (EMBL Heidelberg) using the BamHI (5') and XhoI (3') restriction sites. The new construct was designed to enhance the expression level and solubility of *Cc*HQT with a N-terminal His_6-SUMO3 (small ubiquitin-related modifier) fusion tag (~100 amino acid residues, 11.5 kDa) that has been shown to provide a chaperoning effect (Malakhov *et al.*, 2004). The fusion tag is cleaved off with 1/100 (w/w) SenP2 protease incubated overnight at 4 °C using the elution fraction from the first affinity chromatography step. The robust and specific SenP2 protease is known to recognise the 3-dimensional structure of human SUMO3 rather than a short, but specific, amino acid sequence (Reverter *et al.*, 2006). This procedure introduces only one residue (Ser) at the N-terminus of the expressed protein.

Expression and purification of His_6-SUMO3-CcHQT

[00162] Following the recommended procedure of the Protein Expression and Purification core facility of EMBL-Heidelberg, BL21 (DE3) cells were transformed with pET-

28M-SUMO3-*Cchqt* and grown at 37 °C in LB medium containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol to an OD at λ= 600 nm of 0.5. Expression was induced with 0.2 mM IPTG and cells grown overnight at 18 °C. The cells were harvested by centrifugation and flash-frozen in liquid nitrogen. The bacterial pellets were thawed and resuspended to a final volume of 25 mL/L culture in a lysis buffer composed of 50 mM Tris-HCl pH 8.0, 250 mM NaCl, 20 mM imidazole, Complete Protease Inhibitor, DNaseI and lysozyme. The cells were maintained on ice and ruptured by sonication (Vibra Cell, Bioblock Scientific) with 6 x 25 sec pulses at 60 % intensity and 30 sec breaks. The cell lysate was cleared by centrifugation and the supernatant loaded on to a 5 mL Ni-NTA column (Qiagen). After washing to eliminate unbound contaminants, His_6-SUMO3-HQT was eluted using a gradient of 20 to 300 mM imidazole in the buffer. A protein of 70 kDa that co-elutes with *Cc*HQT from the affinity column may correspond from size comparison to *E. coli* chaperone DnaK (Fig. 11A). The purest fractions were dialysed against 50 mM Tris-HCl pH 8.0 and 250 mM NaCl and digested with 1/100 (w/w) SenP2 overnight at 4 °C. The following day, the digestion mixture was centrifuged and loaded again on the Ni-NTA column. The cleaved *Cc*HQT was found in the flow through and remained stable in solution, while the His_6-SUMO3 fusion was retained on the column. The sample buffer was exchanged for 50 mM Tris-HCl pH 8.0 and 100 mM NaCl, filtered and loaded on a 5 mL HiTrap Q anion-exchange column using an Äkta purifier system. *Cc*HQT was eluted in the flow through (Fig. 11B), while the contaminants remained bound to the MonoQ column. Finally, HQT was analysed by size-exclusion chromatography and eluted as a monomer. The mean yield was 1 mg/L culture of pure *Cc*HQT. The protein was also sensitive to proteolysis and showed a degradation profile similar to that of *Cc*HCT (Fig. 11C). N-terminal sequencing of truncated fragments from HQT degradation gave two sequences: TGPRA and GPRAS, consistent with a cleavage at Lys219 and Thr220. A similar mutant as for HCT, K210A/K219A HQT, was produced and purified using a similar protocol to the native HQT.

HCT and HQT site-directed mutagenesis

[00163] The ability to change specific residues or regions of proteins by site-directed mutagenesis allows a better understanding of structure-function relationships in proteins. Prior to structure determination of the native HCT, several residues potentially involved in catalysis and/ or substrate binding were selected from sequence and structure comparisons

with VS, MaT and TRI101. HCT mutants were designed and generated by site-directed mutagenesis on the *Cchct* gene template to produce H35A, H153A ($\underline{H}X_3D$), H154A ($H\underline{X}X_2D$), D157A ($HX_3\underline{D}$), Y252A, Y255A, R374E and H154N/A155L/A156S ($H\underline{X_3}D$) mutant HCTs.

Table 5: Sequences of the oligonucleotide primers used for the production of mutant HCTs

Mutation	*Pair of mutagenic primers*
c103g/a104c (H35A)	F: atctggtggtgccgaactttgctaccccgagcg (SEQ ID NO:5) R: cgctcggggtagcaaagttcggcaccaccagat (SEQ ID NO:6)
c457g/a458c (H153A)	F: ggtgtgggcatgcgtgctcatgcggcgga (SEQ ID NO:7) R: tccgccgcatgagcacgcatgcccacacc (SEQ ID NO:8)
c460a/t462t (H154A)	F: ggtgtgggcatgcgtcataatgcggcgga (SEQ ID NO:9) R: tccgccgcattatgacgcatgcccacacc (SEQ ID NO:10)
a470c (D157A)	F: catcatgcggcggctggctttagcggc (SEQ ID NO:11) R: gccgctaaagccagccgccgcatgatg (SEQ ID NO:12)
t754g/a755c (Y252A)	F: aagatggcaacaccattagcgctagcagctatgaaatgctgg (SEQ ID NO:13) R: ccagcatttcatagctgctagcgctaatggtgttgccatctt (SEQ ID NO:14)
t763a (Y255A)	F: ccattagctatagcagcaatgaaatgctggccggc (SEQ ID NO:15) R: gccggccagcatttcattgctgctatagctaatgg (SEQ ID NO:16)
c1120g/g1121a/t1122a (R374E)	F: gcattaccagctgggtggaactgccgatccatgatgc (SEQ ID NO:17) R: gcatcatggatcggcagttccacccagctggtaatgc (SEQ ID NO:18)
c460a/g463c/c464t/g466a/c467g/g468c (H154N/A155L/A156S)	F: gggtgtgggcatgcgtcataatctgagcgatggctttagcggcctgcat (SEQ ID NO:19) R: atgcaggccgctaaagccatcgctcagattatgacgcatgcccacaccc (SEQ ID NO:20)

[00164] The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate single site mutations in the codon-optimised *hct*. The double mutants were produced by using the template containing the first mutation and primers corresponding to the second mutation to be introduced. The gene encoding the triple H154N/A155L/A156S mutant HCT was constructed in a single step as the target bases were adjacent. According to the manufacturer's specifications, the reaction mixture was composed of 0.25 µL DNA template, 2.5 µL 10x DNA polymerase buffer, 0.5 µL dNTP, 1 µL forward (F) and 1 µL reverse (R) mutagenic primers at 15 pmol/µL (Table 6), 0.5 µL Pfu Turbo polymerase and completed to 25 µL with milliQ water. After denaturing at 95 °C for 1 min, amplification consisted of 18 cycles of 1 min at 95 °C, 1 min at 60 °C for annealing and 8 min at 68 °C for elongation (1 min/kb of plasmid length). An additional final step of elongation was carried out at 68 °C for 8 min. The PCR products were

digested with 0.5 µL DpnI for 1 h at 37 °C. XL-1 Blue super competent cells (Stratagene) were transformed with 5 µL of the digest. The cells were plated on LB-agar containing the appropriate antibiotic to select colonies containing the mutant plasmid. A few clones were selected on each plate for plasmid DNA preparation and sequencing (Genecore, EMBL-Heidelberg) to identify one that contained the desired mutation. Each mutant was then over-expressed and purified as previously described for the wild-type enzymes. All mutant proteins were successfully produced and purified to homogeneity.

Expression Results

[00165] The production and purification of sufficient quantities of *Cc*HCT, *Cc*HQT, *Cc*4CL2 and *Nt*4CL2 allowed for a detailed characterisation of these acyltransferases and CoA ligases. Some results of biochemical assays performed on *Cc*HCT, *Cc*HQT, *Nt*4CL2 and some of the *Cc*HCT mutants are presented here. Crystallisation trials resulted in crystals of the native and K-mutant HCTs and native *Nt*4CL2. These crystal structures were then solved by MR. *Nt*4CL2 was crystallised in two forms, an apo form and a ternary complex with CoA and AMP bound. Docking of HCAs was carried out in the predicted SBP of the *Nt*4CL2-CoA-AMP ternary complex crystal structure to identify the potential residues involved in catalysis and/ or substrate binding. The overall structure of *Cc*HCT was determined and the native and double mutant structures compared. This was followed by a comparison with the crystal structures of other BAHD acyltransferases. As crystals of *Cc*HCT in complex with its biological substrates could not be obtained, the results of ligand docking experiments using AutoDock Vina are described. A homology-model based on the crystal structure of native *Cc*HCT was constructed and used to compare *Cc*HCT and *Cc*HQT active sites.

Protein over-expression, purification and characterisation

Coffee and tobacco 4-coumarate CoA ligases

[00166] During these investigations of CGA biosynthesis in coffee, the need to generate hydroxycinnamoyl-CoA thioesters was encountered. In a number of biological systems, CoA activation can facilitate the transfer of acylated moieties by acyltransferases. Due to their limited stability, hydroxycinnamoyl-CoA thioesters are not commercially available. Initially, caffeoyl-CoA was synthesised from 5-CQA and CoA using *Cc*HQT in the reverse reaction. But

due to the reversibility of the acyltransfer reaction, only a small amount of CoA thioester was produced. As this procedure was material- and time-consuming, using a 4-coumarate CoA ligase (EC 6.2.1.12) that efficiently catalysed the formation of hydroxycinnamoyl-CoA thioesters was considered. The complete cDNA encoding *Cc*4CL2 was obtained from Nestlé R&D Tours and the recombinant protein produced and purified. It was most likely incorrectly folded as aggregation occurred, and it was inactive towards the biological substrate, coumaric acid. A synthetic gene encoding *Nt*4CL2 was then ordered (Beuerle *et al.*, 2002). This protein was purified using several chromatographic steps with typical yields of 10 mg/L culture and used for further biochemical and structural studies.

Coffee HCT and HQT hydroxycinnamoyltransferases

[00167] Over-expression of these target proteins required the sub-cloning from the pGTP vector into pPROEX_HTb for *hct* and pET-28M-SUMO3 for *hqt*, as well as the screening of a number of different *E. coli* host strains. High yields of N-terminal His_6-tagged *Cc*HCT were obtained with BL21* (DE3) pLysS induced at 20 °C (4.5 mg/L culture). *Coffea canephora* cDNAs contain a high number of rare *E. coli* codons. However, a comparison between the wild-type *Cchct* gene and a codon-optimised gene indicated that the protein expression levels were comparable. The synthetic gene was used in all subsequent protein production, for which an optimised protocol is described above. *Cc*HQT was expressed in BL21 (DE3) cells in fusion with a His_6-SUMO partner, but lower yields were obtained (~1 mg/L culture). HCT and HQT were purified by immobilised metal affinity chromatography (IMAC). The fusion partner or His_6-tag were subsequently cleaved using specific proteases. *Cc*HCT (residues 1-434) plus a five amino acid N-terminal extension (GAMGS) and HQT (1-430) plus a serine introduced at its N-terminus were recovered by subtractive chromatography. Ion-exchange and size-exclusion chromatography were carried out to remove all remaining contaminants. Both *Cc*HCT and *Cc*HQT exist as monomers of 48 kDa in solution and can be concentrated up to 50 mg/mL.

[00168] The native enzymes were partially degraded into two proteolytic fragments of ~25 kDa that appeared during storage at 4 °C and amounts of which increased with time. This may be due to protease contamination from the *E. coli* extract. Limited proteolysis experiments also clearly showed two similar-sized fragments on SDS-PAGE although the fragments remain physically associated throughout size-exclusion chromatography. Mass

spectrometry and N-terminal sequencing analysis showed that the protease cleavage site was located in the predicted cross-over loop region near two lysine residues. To increase the stability of the recombinant *Cc*HCT and *Cc*HQT, trypsin-proteolysis resistant mutants, K210A/K217A *Cc*HCT and K210A/K219A *Cc*HQT, were produced.

II. Biochemical Characterisation

Introduction

[00169] *Nicotiana tabacum* 4-coumarate CoA ligase (*Nt*4CL2) and *Coffea canephora* hydroxycinnamoyl-CoA shikimate/ quinate hydroxycinnamoyltransferases (*Cc*HCT/ *Cc*HQT) were over-expressed in *E. coli* and purified to homogeneity using several chromatographic steps. The following section presents the experimental conditions used to investigate the *in vitro* activity of these enzymes, which are essential components of the biochemical pathway leading to CGAs and other phenolic compounds in plants. *Nt*4CL2 is involved in the synthesis of hydroxycinnamoyl-CoA thioesters, while *Cc*HCT and *Cc*HQT catalyse the transfer of hydroxycinnamoyl moieties between CoA and quinic and shikimic acids. Mutants, notably targeting the conserved HX_3D motif (see below), were also assayed. The acyltransfer leading to CGA biosynthesis is designated as the "forward" reaction, while the "reverse" reaction involves the conversion of a CGA molecule to the corresponding hydroxycinnamoyl-CoA thioester (Fig. 16). The reverse reactions using 5-CQA/ 5-FQA and CoA as substrates were studied first because the CoA thioesters were not commercially available. To study the forward reaction catalysed by *Cc*HCT and *Cc*HQT, these substrates were synthesised enzymatically using *Nt*4CL2 and purified based on a previously published procedure (Beuerle *et al.*, 2002).

Chromatographic Analysis

[00170] To study an enzyme-catalysed reaction, one generally needs to measure substrate depletion and/or product formation. Spectrophotometric methods are useful where either a substrate or a product in the reaction absorb light in a spectral region where other substrates or products do not. In the reactions under study, CoA thioesters and quinate/ shikimate esters have distinct maximum absorbance values, but present overlapping absorption spectra (Fig. 12). Liquid chromatography is more informative about the chemical entities present in the

reaction mixtures under study. It is also more accurate as the peak area of the absorbing species is directly proportional to its concentration. Therefore, high-performance liquid chromatography (HPLC) was preferred for the studies carried out here. Quinic acid is not detectable in the range of 210 to 400 nm wavelength, while shikimic acid can be measured at λ= 210 nm due to the double bond within the cyclohexene ring. High-performance anion-exchange chromatography coupled to pulsed electrochemical detection (HPAE-PED) is another useful technique for the analysis of these acids (Rogers *et al.*, 1999).

[00171] In this study, the analytes were resolved according to their relative hydrophobicity by HPLC on a C_{18}-bonded reverse-phase column (octadecyl hydrocarbon chains anchored to non-polar silica gel particles). A Novapak column (4 µm, 4.6x 250 mm, Waters) or a Nucleosil column (5 µm, 4x 250 mm, Macherey-Nagel) were used in various experiments. Before injection, samples were filtered using a 1 ml syringe and a 13 mm Acrodisc filter with a 0.2 µm membrane (Pall Corporation). A pre-column was used to eliminate compounds that irreversibly bind the C_{18} column and to filter any precipitation that developed between the time samples were loaded for analysis and the actual time of injection by the automatic sampler. Samples were injected into the mobile phase (injection volume of 10 µL, unless otherwise specified) with a flow rate of 0.8 mL/min. The mobile phase consisted of two solvents (A and B) whose relative percentages varied according to the programmed elution gradient. The percentage of organic solvent in the mobile phase was incrementally increased to elute the compounds off the column, the more hydrophobic component eluting last (Table 6). The mobile phase was acidified with 0.1 % phosphoric acid (0.1 % formic acid when coupling with a mass spectrometer) to better separate the ionic and ionisable compounds. The HPLC system used comprised an autosampler (Waters 717), high precision pumps (Waters 600E System Controller), an oven where the column was kept at 30 °C and a Waters photodiode array (PDA) UV detector, scanning wavelengths in the range 210 to 400 nm wavelength. In some experiments, a simpler system equipped with a dual absorbance (DA) detector, but without a column incubator, was used to record the absorbance at two particular wavelengths. Data were processed with the Waters Empower software. Peak assignments were made by comparison with available standards using the peak retention time (RT) and with UV absorbance spectra recorded with the PDA detector.

[00172] The elution gradient used in the initial studies employed acetonitrile. Due to a global acetonitrile shortage in the first half of 2009 (Lowe, 2009), an alternative methanol-based method was designed. Because acetonitrile and methanol differ in their relative eluotropic strength, the elution gradient was modified so as to maintain a good separation of coffee CGA isomers (Table 6).

Table 6: Mobile phase gradients used for HPLC analysis of coffee CGAs

Mobile phase	Acetonitrile		Methanol	
Composition (%)	A:	B:	A:	B:
	8 % CH_3CN	50 % CH_3CN	20 % CH_3OH	60 % CH_3OH
Time (min)	0.1 % H_3PO_4	0.1 % H_3PO_4	0.1 % H_3PO_4	0.1 % H_3PO_4
0	93	7	93	7
2	93	7	93	7
20	90	10	90	10
31	65	35	65	35
40	30	70	30	70
42	30	70	20	80
44	93	7	93	7
55	93	7	93	7

[00173] 5-CQA and rosmarinic acid were available as commercial standards. 5-FQA was provided in limited amounts by Nestlé. A kit of mono- and diCQAs purified from plant extracts was from Biopurify (Chengdu, China). All other chemicals were obtained in a lyophilised form and stored as specified in accordance with recommendations. MilliQ-purified water was used for all reagent dilutions and buffer preparation. Stock solutions were freshly prepared and stored on ice for a maximum of one day, or frozen at -80 °C prior to use. CoA and 5-CQA stock solutions were prepared at 100 mM and 20 mM, respectively. The HPLC-grade solvents (methanol, acetonitrile), as well as the additives (phosphoric and formic acids), were filtered before use. The reaction buffer was prepared using monobasic (0.5 M NaH_2PO_4 at pH 4.0) and dibasic (0.5 M Na_2HPO_4 at pH 9.0) solutions mixed to obtain the appropriate pH value and stored at room temperature.

Table 7: Information on the substrate compounds used in the biochemical assays

Compound	*Formula*	*MW (g/mol)*	*Storage (°C)*	*Min. purity (%)*	*Brand*
5-CQA	$C_{16}H_{18}O_9$	354.31	20	99.1 98	Biopurify Fluka
3-CQA	$C_{16}H_{18}O_9$	354.31	4	99.4	Biopurify
4-CQA	$C_{16}H_{18}O_9$	354.31	4	99.6	Biopurify
5-FQA	$C_{17}H_{20}O_9$	368	4	98	Nestlé
3,5-diCQA	$C_{25}H_{24}O_{12}$	516.46	4	99.3	Biopurify
3,4-diCQA	$C_{25}H_{24}O_{12}$	516.46	4	99.4	Biopurify
4,5-diCQA	$C_{25}H_{24}O_{12}$	516.46	4	99.1	Biopurify
caffeic acid	$C_9H_8O_4$	180.16	20	98	Sigma
ferulic acid	$C_{10}H_{10}O_4$	194.19	20	99	Sigma
t-cinnamic acid	$C_9H_8O_2$	148.16	20	99	Sigma
coumaric acid	$C_9H_8O_3$	164.16	20	98	Sigma
sinapic acid	$C_{11}H_{12}O_5$	224.21	20	98	Sigma
coenzyme A	$C_{21}H_{36}N_7O_{16}P_3S$	767	-20	85 96	Sigma BioChemika
quinic acid	$C_7H_{12}O_6$	192	20	98	Fluka
shikimic acid	$C_7H_{10}O_5$	174	20	99	Sigma
rosmarinic acid	$C_{18}H_{16}O_8$	360.3	20	97	Aldrich
spermidine	$C_7H_{19}N_3$	145.25	20	99.5	Sigma
myo-inositol	$C_6H_{12}O_6$	180.2	20	99.5	Sigma
pHPL	$C_9H_{10}O_4$	182.2	20	98	Aldrich

[00174] The retention time (RT) and absorbance spectra was determined for each of the HCAs that serve as substrates for 4CL2 (Table 8). Caffeic, coumaric, ferulic and sinapic acids were injected individually and analysed by HPLC with either methanol or acetonitrile as the organic solvent.

Table 8:Typical retention times and absorbance maxima of the HCA standards

Compound	*RT (min)*		*Max. abs. (nm)*
	methanol	*acetonitrile*	
Coumaric acid	31.9	28.9	308.3
Caffeic acid	18.3	16.7	322.6
Ferulic acid	37.8	35.2	322.6
Sinapic acid	40.2	38.2	322.6

[00175] CoA eluted close to the void volume and was best measured at λ= 256 nm (Table 9). As can be seen in Table 9, the CGA elution sequence, which was driven by an increase in hydrophobicity, did not change in the chromatographic systems used (3-CQA< 4-CQA< 5-CQA< 5-FQA< 3,4-diCQA< 3,5-diCQA< 4,5-diCQA). The UV spectrum of each CQA isomer was very similar. RTs for each compound could vary depending on the mobile phase (acetonitrile/ methanol), HPLC device (photodiode array or dual absorbance detector), or column (Nucleosil/ Novapak) used (Table 9). Pure CGAs were systematically injected as standards either individually or in a mixture before each reaction series. As an alternative to pure commercial standards, methanolic extracts of green coffee beans proved to be a convenient source of the major coffee CGAs. Typically, the beans were frozen in liquid nitrogen then ground. Phenolic compounds were extracted with 70 % methanol for 1 h at 40 °C in shaking flasks. The methanolic extract was filtered (0.2 µm) and a 10-fold dilution was analysed by HPLC-PDA (data not shown).

Table 9: Relative retention times and absorbance maxima of the CGA standards in the HPLC system

Compound	*RT (min)*		*Maximum absorbance (nm)*
	methanol	*acetonitrile*	
CoA	4.7	3.9	256.0
3-CQA	6.8	6.8	325.0
4-CQA	12.0	11.9	326.2
5-CQA	13.8	13.0	325.0
5-FQA	29.3	27.1	325.0
3,4-diCQA	45.6	42.1	325.0
3,5-diCQA	47.2	43.1	328.6
4,5-diCQA	50.6	44.5	328.6

[00176] A calibration of peak area against concentration was obtained for 5-CQA at λ= 325 nm. Linearity was respected in the range 0 to 10 mM (data not shown). To quantify CGAs in a coffee methanolic extract, a reference solution of 5-CQA at a known concentration was analysed as an external standard before the sample of interest was tested. The concentration of CGAs in the sample was determined from the corresponding peak area at λ= 325 nm and the values for the 5-CQA standard (Equation 1). The relative response factor (RRF) was taken into account as a correction for molecular weight differences with the 5-CQA standard (Lepelley *et al.*, 2007).

Equation 1: Quantification of CGA compounds with 5-CQA as an external standard.

$$A_{std}/C_{std} = A_{5\text{-}CQA}/C_{5\text{-}CQA} = A_{CGA}/C_{CGA} * RRF$$

where A: absorbance; C: concentration; RRF: relative response factor: RRF= 1 for mono-CQAs, 0.96 for 5-FQA and 1.36 for diCQAs.

[00177] The stability of CGA compounds was verified by heating 10 mM 5-CQA/ 5-FQA in 0.1 M sodium phosphate pH 6.0/ 7.0 at 90 °C. Samples taken at t= 0, 30 and 60 min were analysed by HPLC-DA. Similar experiments were performed with 1 mM 3-/ 4-/ 5-CQA, 3,4-/ 3,5-/ 4,5-diCQA in 0.1 M sodium phosphate pH 6.5 incubated overnight (~15 h) at 34/ 25 °C. Samples (1369-1386) were analysed using HPLC-PDA with acetonitrile.

[00178] Stock solutions of substrates and buffer were mixed with MilliQ water to reach the desired concentration and final volume. Reactions were started by adding the enzyme (or water for the control reaction) and solutions incubated in a 34 °C water-bath during sampling, unless otherwise stated. Typically, a 40 µL aliquot was taken and diluted with 210 µL HPLC solvent A. After filtration, the automated system injected a 10 µL sample into the HPLC system. The following sections describe the enzymatic reaction details. The figure legends in the Results section of this thesis also contain reaction details and sample numbers. All chromatograms reported represent, as a function of time, the absorbance value measured at λ= 325 nm, except where otherwise stated.

Enzymatic synthesis and purification of the CoA thioesters

Coffee and tobacco 4-coumarate CoA ligases

[00179] To assay the activity of *Cc*4CL2, a pure fraction of the recombinant protein (5 µM) was incubated with 0.2 mM coumaric acid, its major biological substrate, in the presence of 1 mM CoA, 5 mM $MgCl_2$ and 5 mM ATP in 0.1 M sodium phosphate pH 7.5. Samples (876-879) taken at t= 0, 15 min and after overnight incubation at 34 °C were analysed using HPLC-PDA with methanol.

[00180] The activity of *Nt*4CL2 was tested with cinnamic acid and its common derivatives coumaric, caffeic, ferulic and sinapic acids. Reaction mixtures containing 1 mM (hydroxy)cinnamic acid, 5 mM CoA, 2.5 mM $MgCl_2$ and 2.5 mM ATP in 0.1 M Tris-HCl pH 7.5 were prepared in a 400 µL final volume. Reactions were started by adding 1.6 µM recombinant enzyme. Samples (590-603) taken at t= 0, 2 and 20 min were analysed using HPLC-PDA with

methanol. Similar reaction mixtures were made up in three different buffers: 0.1 M sodium phosphate pH 6.0/ 7.5 and 0.1 M Tris-HCl pH 7.5. Samples (634-683) taken at t= 0, 5, 25 min and after overnight incubation were analysed using HPLC-PDA using methanol.

[00181] Because commercial CoA thioesters were not available, coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA were synthesised enzymatically as follows: 10 mL reaction mixtures composed of 0.8 mM coumaric/ caffeic/ ferulic acid, 1 mM CoA, 2 mM $MgCl_2$ and 2 mM ATP were prepared in 0.1 M sodium phosphate pH 6.0. Reactions were started by adding 0.05 µM *Nt*4CL2. Samples (699-715) taken at t= 0, 10, 60 and 150 min were filtered through a 50 kDa cut-off membrane to remove the enzyme. The filtrates were analysed using HPLC-PDA with the methanol method. The reaction was complete after 150 min and all subsequent reactions to produce the hydroxycinnamoyl-CoA thioesters were stopped at this time. The final concentration of the CoA thioester was deduced from the initial amount of HCA supplied in the reaction.

Purification of CoA thioesters

[00182] Successive injections of reaction volumes of up to 150 µL, corresponding to the maximum loop capacity, were carried out to purify caffeoyl-CoA by preparative HPLC using either acetonitrile or methanol as described above. The reaction product was subsequently collected manually as it eluted off the column.

[00183] Based on a previously described procedure (Beuerle *et al.*, 2002), a simpler method to purify CoA thioesters from a reaction mixture was developed. A 1 mL solid-phase extraction (SPE) Sep-Pak C_{18} cartridge (Waters) was activated with 10 column volumes (CV) of methanol, washed with 10 CV milliQ H_2O and equilibrated with 10 CV of 4 % ammonium acetate buffer. Ammonium acetate was added to the reaction mixture to a final concentration of 4 %, before loading onto the cartridge using a syringe. The column was subsequently washed with 10 CV of 4 % ammonium acetate and 10 CV of milliQ water. The CoA thioesters were then eluted with several 500 µL fractions of 100 % methanol, which were analysed by HPLC.

[00184] The fractions containing the CoA thioester collected from preparative HPLC or eluted from the SPE column were placed in 1.5 mL Eppendorf tubes and evaporated to

near dryness using a vacuum concentrator. The residue was resuspended in milliQ-purified water before further HPLC analysis or use in enzymatic reactions.

Comparison of the HCT and HQT-catalysed forward reactions

HCT and HQT acyl acceptor specificity

[00185] In the forward direction, HCT and HQT catalyse the transfer of hydroxycinnamoyl moieties from CoA to quinic or shikimic acid. To verify the substrate preferences, reaction mixtures containing 5 mM quinic/ shikimic acid or both and 0.2 mM caffeoyl-CoA in 0.1 M sodium phosphate pH 6.5 were prepared in a final volume of 100 µL. Reactions were started by adding 1 µM HCT/ HQT. Samples (1023-1052) taken at t= 0, 5 and 60 min were analysed using HPLC-PDA with the methanol method.

[00186] To test the reactivity of HCT/ HQT towards other acyl acceptor molecules, reactions containing 10 mM quinic/ shikimic/ 4-hydroxyphenyllactic acid/ spermidine or *myo*-inositol and 0.2 mM caffeoyl-CoA in 0.1 M sodium phosphate pH 6.5 were prepared in a 100 µL final volume. Reactions were started by adding 0.5 µM HCT/ HQT. Samples (1146-1163) taken at t= 0, 15 min and after overnight incubation were analysed using HPLC-PDA with the methanol method.

HCT and HQT phenolic donor specificity

[00187] To compare the phenolic donor preference of HCT and HQT in the forward reaction, the following assays were set up. Reaction mixtures containing 0.2 mM caffeoyl-CoA/ coumaroyl-CoA/ feruloyl-CoA and 10 mM quinic/ shikimic acid in 0.1 M sodium phosphate pH 6.0 were prepared in a 500 µL final volume. Reactions were started by adding 0.4/ 0.004 µM HCT/ HQT. Samples (716-727, 764-775 and 800-811) taken at t= 0, 10, and 75 min were analysed with the methanol method.

Comparison of the HCT and HQT-catalysed reverse reactions

Characterisation of the products obtained in the reverse reaction

[00188] The activity of recombinant HCT and HQT was first tested towards the CGAs available, 5-CQA and 5-FQA, with CoA as the cofactor. Reaction mixtures containing 3.75 mM 5-CQA/ 5-FQA and 5 mM CoA in 0.1 M sodium phosphate pH 6.0 were prepared in a

final volume of 100 µL. Reactions were started by adding 1 µM HCT or 1/ 0.1/ 0.01 µM HQT. Samples (395-428) taken at t= 0, 5, 15, 30, and 60 min were analysed using HPLC-PDA with the methanol method.

[00189] To confirm the formation of caffeoyl- and feruloyl-CoA, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed. For this, reaction mixtures made up of 2 mM 5-CQA/ 5-FQA and 2 mM CoA in 0.1 M sodium phosphate buffer pH 6.0 were prepared to a final volume of 500 µL. Reactions were started by adding 0.04 µM HQT and incubated at 25 °C. Samples taken at t= 0 and 45 min for 5-CQA (sample 3) and t= 0 and 60 min for 5-FQA (sample 4) were analysed using HPLC-PDA with the acetonitrile method. These samples were also sent to the Nestlé Research Centre, (Lausanne) for LC-MS/MS analysis. The negative detection mode was adopted because phenolic acids ionise better in this mode.

[00190] The Biopurify kit enabled the testing of the activity of HCT and HQT towards other CGA monoesters (3-CQA and 4-CQA) and diesters (3,4-diCQA, 3,5-diCQA and 4,5-diCQA). Reaction mixtures comprised 1 mM CGA and 1/ 5 mM CoA in 0.1 M sodium phosphate pH 6.0/ 7.5. Reactions were started by adding 0.4 µM HCT/ HQT. Samples (604-633 and 684-698) were taken at t= 0, 30 min and after overnight incubation and analysed using HPLC-PDA with the methanol method.

[00191] Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, similar in nature to CGAs. The reactivity of HCT and HQT was therefore tested towards this potential substrate. A reaction mixture composed of 1 mM rosmarinic acid and 1 mM CoA in 0.1 M sodium phosphate pH 6.5 was prepared in a 100 µL final volume. Reactions were started by adding 1 µM HCT/ HQT. Samples (978-987) were taken at t= 0 and after overnight incubation and analysed using HPLC-PDA with the methanol method.

Temperature and pH influence

[00192] Initial characterisation of the optimal pH and temperature conditions was carried out with a reaction mixture composed of 2 mM 5-CQA, 2 mM CoA and 1 µM HCT/ HQT in 0.1 M sodium phosphate buffer. The temperature (22, 34 and 42 °C at pH 6.0) and pH values (4.6, 6.0 and 7.6 at 34 °C) were varied. Samples (36-47 and 49-58) were analysed by HPLC-PDA using methanol.

Influence of enzyme, 5-CQA, CoA and quinic acid concentrations

[00193] To study the influence of 5-CQA concentration, reaction mixtures containing 0.02 to 10 mM 5-CQA and 2 mM CoA in 0.1 M sodium phosphate pH 7.0 were prepared in a 500 µL final volume. Reactions were started by adding 0.1 µM HQT and incubated at 30 °C. Samples taken at t= 0 to 30 min were analysed using HPLC-DA with acetonitrile.

[00194] To determine the influence of enzyme concentration, reaction mixtures containing 2 mM 5-CQA and 5 mM CoA in 0.1 M sodium phosphate pH 6.0 were prepared in a 500 µL final volume. Reactions were started by adding various concentrations of HCT/ HQT and incubated at 30 °C. Samples taken at t= 0 to 60 min were analysed using HPLC-DA with acetonitrile.

[00195] The influence of quinic acid concentration was studied using reaction mixtures containing 5 mM CoA, 5 mM 5-CQA and 0/ 1/ 5/ 20 mM quinic acid in 0.1 M sodium phosphate pH 6.0 in a 100 µL final volume. Reactions were started by adding 1 µM HQT. Samples (488-503) taken at t= 0, 5, and 30 min for were analysed using HPLC-PDA with methanol.

[00196] To study the influence of CoA, varied concentrations of CoA (0.125, 0.25, 0.5, 1 and 5 mM) were added to 1 mM 5-CQA in 0.1 M sodium phosphate pH 6.5 to a 500 µL final volume. Reactions were started by adding 0.5 µM HCT/ HQT. Samples (1101-1145 and 1164-1168) taken at t= 0, 3, 30, 180 min, and after overnight incubation were analysed using HPLC-PDA with acetonitrile.

Comparison of the activity of native and mutant HCTs

Activity assay in the forward and reverse reactions

[00197] To determine the influence of point mutations on HCT enzymatic activity, 0.1 mM caffeoyl-CoA, 1 mM quinic/ shikimic acid or both were mixed in 0.1 M sodium phosphate pH 6.5 to a 100 µL final volume. Reactions were started by adding 5 µM native, K210A/K217A (K), H153A, H35A, D157A, Y252A, Y255A, R374E, H154A and H154N/A155L/A156S mutant HCTs. Samples (1321-1350) taken at t= 0 and 210 min were analysed using HPLC-PDA with methanol as the organic solvent.

Additional analysis of the H154A and H154N/A155L/A156S mutants

[00198] To study in more detail the activity of the mutants targeting H154, reaction mixtures containing 5 mM 5-CQA and 5 mM CoA in 0.1 M sodium phosphate pH 6.0 were prepared in a 100 µL final volume. Reactions were started by adding 1 µM native, H154A or H154N/A155L/A156S mutant HCTs. Samples (326-349, 464-487, 956-977 and 1023-1052) were taken at various time points and analysed using HPLC-DA/ PDA with methanol or acetonitrile.

[00199] Caffeoyl-CoA (0.01-0.2 mM) was similarly incubated with 1 mM 5-CQA and the native or H154A mutant HCTs. Samples (1169-1193) taken at t= 0, 15 min and after overnight incubation were analysed using HPLC-PDA with the acetonitrile method.

[00200] In addition to the assays above, the H154A and H154N/A155L/A156S HCT mutants were incubated with various acyl donor and acceptor moieties. In this case, reaction mixtures consisting of 10 mM 5-CQA/ 5-FQA and 0.2 mM CoA/ caffeoyl-CoA/ feruloyl-CoA/ coumaroyl-CoA in 0.1 M sodium phosphate pH 6.5 were prepared in a 200 µL final volume. Reactions were started by adding 0.5 µM native or variant HCT. Samples (1194-1273) taken at t= 0, 60 min and after overnight incubation were analysed using HPLC-PDA with acetonitrile.

Characterization of Substrate Binding Pockets (SBP) for 4CL2, HCT, and HCQ.

[00201] Substrate docking studies revealed that the substrate binding pocket (SBP) of *Nt*4CL2 could be clearly defined as part of the N-terminal domain with CoA thiol and AMP phosphate group at the entry of the pocket. Amino acid residues within 4 Å of the predicted SBP are: Gln213, Leu221, Ile238, Tyr239, Ser243, Val244, Pro279, Met306-Ala309, Gly330-Thr336, Pro340, Val341 and Phe348. (Data not shown). These residues in *Nt*4CL2, and corresponding residues in related 4CL enzymes may be useful targets for altering the enzymatic activity with respect to one or more substrates or products.

[00202] Pocket-Finder (Hendlich *et al.*, 1997) was used to predict the location of the SBP in the native *Cc*HCT crystal structure chain A, which most likely adopts a functional conformation (see Results section). The 1.6 Å probe radius used found a major site (volume~ 1850 $Å^3$) corresponding to the solvent channel located at the domain interface (data not shown). The residues lining the solvent channel in HCT were identified. They belong to the following

sequence regions: Asn26-Tyr40, Pro110, Arg115, Val149-Ile165, Tyr203, Tyr255, Tyr281-Asp285, Arg289, Asn301-Thr305, Leu331, Leu346, Leu355-Phe362, Gly368-Ile377, Met390-Tyr397 and Leu400-Phe402. A number of these residues are conserved between the HCT and HQT enzymes, while some are not. These differences may contribute to the different substrate specificities of HCT and HQT.

Characterisation Results

[00203] Reverse-phase HPLC using an elution gradient with either acetonitrile or methanol was the preferred technique for the characterisation and analysis of plant secondary metabolites in the biochemical characterisation of the enzymes produced herein. The HPLC were adjusted to optimise CGA separation. A preparative HPLC procedure was developed for the purification of caffeoyl-CoA from enzymatic reactions. However, a simpler SPE method was ultimately used for the purification of coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA esters, which are substrates for HCT and HQT in the forward reactions leading to CGA biosynthesis.

Enzymatic synthesis and purification of CoA thioesters

Coffee and tobacco 4-coumarate CoA ligases

[00204] Enzymatic synthesis was found to be an efficient way of producing large amounts of CoA-activated HCAs that are not commercially available. The well-characterised *Nt*4CL2 was produced using a published procedure (Beuerle *et al.*, 2002). With all substrates used, apart from sinapic acid, there is a high conversion of HCA to the relevant CoA thioester. The retention time and maximum absorbance values in absorbance spectra are presented in Table 10. Fig.13 shows the activity of purified recombinant 4CL2 towards cinnamic, coumaric, caffeic, ferulic and sinapic acids in the presence of CoA and Mg-ATP required for catalysis.

Table 10: Relative retention times and absorbance maxima of CoA thioester products

Compound	*RT (min)*		*Max. abs. (nm)*
	methanol	*acetonitrile*	
Cinnamoyl-CoA	50.1	-	306.0
Coumaroyl-CoA	43.1	42.4	333.3
Caffeoyl-CoA	34.4	34.6	348.9
Feruloyl-CoA	44.4	44.1	347.7
Sinapoyl-CoA	45.3	45.4	351.3

[00205] Hydroxycinnamoyl-CoA thioesters were characterised by their UV spectra (Fig. 14). Later experiments show that, with the exception of sinapic acid, the conversion by *Nt*4CL2 of these substrates reached over 80 % after 60 min (Fig. 15). During optimisation of the reaction conditions, no major difference was observed in product levels obtained at pH 6.0 or 7.5, or with sodium phosphate compared to Tris-HCl (data not shown). Therefore all subsequent reactions were carried out in 0.1 M sodium phosphate pH 6.0. The CoA esters could be produced almost free of their acid precursors by the addition of more enzyme. This further enabled the quantification of the CoA thioester product, assuming that one mole of HCA supplied in the reaction generated one mole CoA thioester.

Purification of CoA thioesters by preparative HPLC or SPE

[00206] The coumaroyl-, caffeoyl- and feruloyl-CoA thioesters produced were purified by preparative HPLC or solid phase extraction (SPE). Initially, preparative HPLC was used for purification. A 100-150 µL volume of reaction mixture was injected and the fractions containing the CoA ester were collected manually. This purification method was abandoned because it was time-consuming and the HPLC profiles obtained indicated a partial degradation of caffeoyl-CoA to caffeic acid. This was most likely due to the increase in relative amounts of phosphoric acid during concentration. A simpler Sep-Pak purification procedure was eventually devised. The enzyme was removed from the reaction mixture by filtration through a 50 kDa membrane before loading onto a Sep-Pak column. The CoA thioesters were bound to the C_{18} column using a 4 % ammonium acetate buffer, washed with milliQ water and eluted with methanol. The elution fractions were collected and analysed by HPLC. The fractions containing the CoA ester were lyophilised and resuspended in milliQ water before use.

Comparison of the HCT and HQT-catalysed forward reactions

Test of the acyl acceptor specificity

[00207] Coumaroyl-, caffeoyl- and feruloyl-CoA thioesters were produced using *Nt*4CL2, and then purified. The purified thioesters were substantially free from their acid precursors. The reactivity of *Cc*HCT and *Cc*HQT towards these activated substrates was tested. HCT and HQT can transfer the acyl moiety between CoA and acceptor molecules such as quinic and shikimic acids. The precursor molecule for the major CGA compound, 5-CQA, is caffeoyl-

CoA, which may be an important precursor in mono- and dicaffeoylquinic acid biosynthesis. Little was known about the synthesis of coumaroylquinic or feruloylquinic acids in plants. However, coumaroyl-CoA is the precursor for coumaroylquinic acids, which are present only in trace amounts in coffee. Feruloyl-CoA appears to be the precursor for 5-feruloylquinic acid (5-FQA).

Activity of CcHCT and CcHQT towards caffeoyl-CoA

[00208] The results concerning the reactions containing caffeoyl-CoA and catalysed by HCT are presented in Fig. 17. When *Cc*HCT was incubated with caffeoyl-CoA and quinic acid, 5-CQA (RT= 17.6 min) was formed. 76 % of the caffeoyl-CoA substrate had been consumed after 60 min respectively (Panel B). With shikimic acid as the acyl acceptor, all the caffeoyl-CoA present was transformed after only 5 min (Panel C) to a product (RT= 35.4 min) with a typical CGA absorbance spectrum (Panel D). The UV spectrum showed a maximum at λ= 322.6 nm and is similar to that of 5-CQA. As caffeoyl-CoA was used as a precursor, and the absorption spectrum of the novel compound shared some properties with that of 5-CQA, it was hypothesised that the novel compound resulted from a coupling of the caffeoyl moiety with shikimic acid via an ester linkage, presumably caffeoylshikimic acid (CSA). After 60 min, a major peak representing the same ester and two other peaks, which may correspond to the two other CSA isomers (RT= 25.9 and 32.2 min) were present (Panel D). When both quinic and shikimic acids were supplied to *Cc*HCT at the same concentration, a caffeoylshikimic acid isomer was formed after 5 min (Panel E); after 60 min, small peaks corresponding to the two other CSA isomers were detected as well as a small peak for 5-CQA (Panel F). This result clearly demonstrates that *Cc*HCT strongly favours shikimic over quinic acid as the acyl acceptor molecule and is capable of producing more than one CSA isomer.

[00209] *Cc*HQT was incubated with caffeoyl-CoA and quinic or shikimic acid (Fig. 18). With quinic acid as a substrate, caffeoyl-CoA was converted to 5-CQA (RT= 17.5 min). As for *Cc*HCT, incubation with caffeoyl-CoA and shikimic acid resulted in the accumulation of a CSA (RT= 35.5 min) (Fig. 18B). Conversely, when both quinic and shikimic acids were supplied to *Cc*HQT, the enzyme mainly formed 5-CQA and only a small amount of CSA after t= 5 min (Fig. 18C). After 60 min, 5-CQA and CSA are present in much closer ratios,

57 and 43 % respectively (Fig. 18D). This indicates that *Cc*HQT prefers quinic acid, but is also capable of forming CSA.

[00210] The activity of *Cc*HCT and *Cc*HQT towards other potential substrates (4-hydroxyphenyllactic acid, spermidine and *myo*-inositol) was also assayed. These acceptor molecules were incubated with caffeoyl-CoA and the enzymes, however no activity was observed (data not shown). *Cc*HCT and *Cc*HQT are therefore active only towards the alicyclic quinic and shikimic acids.

Activity of HCT and HQT towards coumaroyl-CoA

[00211] The incubation of coumaroyl-CoA and quinic acid with *Cc*HCT resulted in a peak (RT= 27.2 min) presumably corresponding to a coumaroylquinic acid (Fig. 19B). With shikimic acid, three peaks were detected at RT= 33.2, 38.5 and 42.1 min, presumably representing 3-, 4- and 5-coumaroylshikimic acids (Fig. 19C). When both acyl acceptors were present, a mixture of the three coumaroylshikimic acids plus a small amount of coumaroylquinic acid was observed (Fig. 19D). So with coumaroyl-CoA, CcHCT appears to prefer shikimic acid as an acyl acceptor and produces more than one coumaroylshikimic acid isomer.

[00212] *Cc*HQT formed a coumaroylquinic acid from coumaroyl-CoA and quinic acid (Fig. 20A). Coumaroyl-CoA and shikimic acid incubated with *Cc*HQT resulted in only one coumaroylshikimic acid isomer at RT= 41.9 min (Fig. 20B). When both quinic and shikimic acids were supplied, HQT synthesised a mixture of presumably coumaroylquinic and coumaroylshikimic acid in the ratio of 85 and 15 % respectively at t= 10 min (Fig. 20C). This ratio shifted to 78 and 22 % at t= 75 min (data not shown). As seen when using caffeoyl-CoA as the forward substrate, it is again noted that *Cc*HQT prefers quinic acid and produces only one coumaroylshikimic acid isomer.

Activity of CcHCT and CcHQT towards feruloyl-CoA

[00213] *Cc*HCT can synthesise a shikimate ester, presumably a feruloylshikimic acid (RT= 45.2 min) from feruloyl-CoA and shikimic acid and the reaction was complete after 2 min. The new compound has a typical CGA absorbance spectrum (Fig. 21B). However, only a small peak of 5-FQA (RT= 30.5 min) is formed in the presence of quinic acid at t= 20 min (Fig. 21C). When both acyl acceptors were supplied, *Cc*HCT catalyses the synthesis of a

feruloylshikimic acid corresponding to the RT for the 5-acyl isomer (Fig. 21D). This demonstrates the preference of CcHCT for shikimic acid and its much lower efficiency towards 5-FQA formation.

[00214] The incubation of feruloyl-CoA with HQT and quinic acid leads to 5-FQA (RT= 30.5 min) (Fig. 22A), while with shikimic acid it leads to a feruloylshikimic acid (RT= 45.1 min) (Fig. 22B). The conversion was complete after t= 2 min and 20 min respectively. As expected, when both quinic acid and shikimic acid were supplied, HQT preferentially synthesised 5-FQA (Fig. 22C).

[00215] In summary, in the presence of the same CoA thioester (caffeoyl-/ coumaroyl-/ feruloyl-CoA), *Cc*HCT clearly prefers shikimic over quinic acid. *Cc*HCT is also much less efficient with feruloyl-CoA in the presence of quinic acid. In contrast, *Cc*HQT is able to use all three CoA thioesters equally with both quinic and shikimic acids. It is also interesting to note that HCT can form three probable shikimate esters of caffeic and coumaric acids, while with feruloyl-CoA only a single shikimate ester is formed. In contrast, *Cc*HQT forms only a single shikimate ester with all CoA thioesters tested.

Comparison of the HCT and HQT-catalysed reverse reactions

Stability of chlorogenic acids

[00216] CGAs are known to be unstable at basic pH and stable at acidic pH (Friedman, 2000). This was verified by heating buffered solutions of 5-CQA and 5-FQA to 90 °C. At pH 6.0, significant amounts of 3- and 4-CQA were produced from the 5-CQA isomer. At neutral pH, 5-CQA was converted to approximately equivalent levels of 3- and 4-CQA isomers, resulting in an equimolar mixture of the three caffeoylquinic acid isomers (Table 11). No major difference was observed between 30 min and 60 min incubation times (data not shown). 5-FQA behaved very similarly to 5-CQA by partial isomerisation to 3- and 4-FQA (data not shown). Further experiments performed at 34 and 25 °C on the mono- and diCQAs from Biopurify showed that, even though the enzymatic reactions were carried out at 34 °C and pH 6.5, 5-CQA partially isomerised into 4-CQA and to a lesser extent into 3-CQA (Fig. 23C and C'). In a 4-CQA solution, the acyl migrated to the 3-hydroxyl position and to a lesser extent to the 5-hydroxyl position (Fig. 23B, B'). 3-CQA isomerised into 4-CQA and, to a lesser extent, into 5-CQA (Fig.

23A, A'). Similarly, 3,5-diCQA isomerised into 3,4- and 4,5-diCQA when incubated overnight at 25 °C and this isomerisation increased when the sample was kept at 34 °C (data not shown).

Table 11: Effect of temperature and pH on the isomerisation of 5-CQA 10 mM 5-CQA in 0.1 M sodium phosphate ph 6.0 or 7.0.

pH	*Treatment*	*Isomeric ratio (%)*		
		5-CQA	4-CQA	3-CQA
6.0	90 °C for 30 min	65.0	19.6	15.4
7.0	90 °C for 30 min	32.4	31.3	36.3

[00217] This isomerisation most probably occurs via the acyl transfer of the hydroxycinnamoyl moiety between the three readily accessible hydroxyl positions of quinic acid (hydroxyl at C-1 is not involved). In some of the enzymatic reactions that will be described later, a significant amount of 3- and 4-CQA isomers was derived from solutions containing 5-CQA, especially after overnight incubation. It is assumed that this occurred not from enzymatic synthesis but from chemical isomerisation.

Characterisation of the reverse reaction products formed by HCT and HQT

[00218] When CoA was added as a substrate with 5-CQA in the presence of *Cc*HCT or *Cc*HQT, there was the emergence of a peak at RT= 27.5 min showing a characteristic CoA thioester UV spectrum (Fig. 24B, C), presumably corresponding to caffeoyl-CoA. However, under identical reaction conditions and enzyme concentrations, *Cc*HCT (Fig. 24B) produces significantly less caffeoyl-CoA than HQT (Fig. 24C). A 100-fold dilution of HQT can synthesise the same amount of caffeoyl-CoA as HCT at a 1 μM concentration (Fig. 24B, D). This is further evidence that CcHQT has a greater preference for reactions involving quinate moieties than does CcHCT.

[00219] Further evidence of this is that no product formation was observed when *Cc*HCT was incubated with 5-FQA and CoA under the conditions tested (Fig. 25A, B) and that, in contrast, *Cc*HQT was with the same substrates able to synthesise a product at RT= 39.8 min, which probably corresponds to feruloyl-CoA, as it shows a UV spectrum characteristic of a CoA thioester (Fig. 25C).

[00220] The main reaction products when using 5-CQA or 5-FQA as substrates were presumed to be CoA thioesters. The absorption spectra of the compounds contained in the HPLC

peaks measured using HPLC-PDA present two maxima around 256 and 346 nm, the latter being characteristic of the presence of a thioester bond. The compounds were subjected to a LC-MS/MS analysis to confirm their identity. An m/z value of 928 for the reaction product synthesised by *Cc*HQT from 5-CQA and CoA corresponds to caffeoyl-CoA (MW= 930 g/mol). An m/z value of 942 for the reaction product synthesised by HQT from 5-FQA and CoA corresponds to feruloyl-CoA (MW= 944 g/mol).

Substrate specificity of HCT and HQT in the reverse reaction

[00221] For preliminary experiments testing *Cc*HCT and *Cc*HQT specificity (data not shown), various CGA isomers were generated by heating solutions of 10 mM 5-CQA/ 5-FQA buffered at pH 7.0. Only the 5-CQA peak was clearly decreased by conversion to caffeoyl-CoA with *Cc*HCT and *Cc*HQT in the presence of CoA. No activity towards 3-CQA and 4-CQA was detected. Similarly, with a FQA mixture only the 5-FQA isomer peak was decreased in the presence of *Cc*HQT. HCT was found to be less efficient towards this substrate. When the CQA kit was obtained from Biopurify, reaction mixtures were prepared with each monoester individually and CoA. Again, only 5-CQA was converted by *Cc*HCT and *Cc*HQT to caffeoyl-CoA in the presence of CoA (Fig. 26), confirming the results obtained with the CQA isomer mixture. Rosmarinic acid was also incubated overnight with CoA, but no reaction product was observed with *Cc*HCT or *Cc*HQT (data not shown).

[00222] *Cc*HCT and *Cc*HQT were also incubated with 3,5-diCQA and CoA. In the control with no enzyme, 3,5-diCQA isomerises into 3,4-diCQA and possibly 4,5-diCQA, however, this peak could not be resolved from the 3,5-diCQA peak at RT= 47.2 min (Fig. 27B). In the presence of *Cc*HCT, 5-CQA and caffeoyl-CoA are detected as indicated from the 5-CQA standard and the absorbance spectra (Fig. 27A, C). In the presence of *Cc*HQT, no reaction product was observed after overnight incubation (data not shown).

Additional enzymatic properties of HCT/ HQT

[00223] Additional experiments were conducted to further study the reaction properties of *Cc*HCT and *Cc*HQT. Temperature variation (22 °C, 34 °C and 42 °C) did not significantly alter the activity of *Cc*HCT and *Cc*HQT in the reverse reaction with 5-CQA and CoA. All subsequent enzymatic reactions were therefore carried out in a 34 °C water-bath.

[00224] To address the effects of pH on the reverse reactions of *Cc*HCT and *Cc*HQT, several experiments were carried out. In the first set of reactions performed, *Cc*HQT and *Cc*HCT were not active at pH 4.6 (Fig. 28). The results from a more extensive analysis on the effects of pH on the reverse reaction confirmed that the *Cc*HQT optimal pH value is close to 6.0, whereas *Cc*HCT seemed to be more active towards 5-CQA at pH 8.0 (Fig. 29). pH values higher than pH 8.0 have not yet been tested with either enzyme because CGA compounds are not stable at this pH. These results also highlight the remarkable difference in the catalysis efficiency of *Cc*HCT and *Cc*HQT with similar levels of 5-CQA and CoA substrates and confirm the preference of *Cc*HQT for quinate substrates.

Effect of enzyme and substrate concentrations on product formation

[00225] As seen above, the production of the same amount of caffeoyl-CoA (at constant 5-CQA and CoA concentrations) requires very different amounts of HCT and HQT. The reaction steady-state persists for many minutes with HCT in the range of concentrations that were used (0.1-0.8 μM) (Fig. 30A). Under these conditions, the Michaelis-Menten law may be applicable (Bisswanger, 2002). The reaction velocity as a function of substrate and enzyme concentrations can yield the Michaelis and catalytic constants. However, lower enzyme concentrations are required in the case of HQT (Fig. 30B).

[00226] When serial dilutions of 5-CQA were incubated with CoA and *Cc*HQT, a maximal conversion to caffeoyl-CoA production is reached very rapidly, which corresponds to the equilibrium attained between substrates and products (Fig. 31).

[00227] To study the potential effect of quinic acid concentration on the *Cc*HQT-catalysed reverse reaction, initial quinic acid concentrations were varied from 0 to 20 mM in the presence of constant levels of 5-CQA (5 mM) and CoA (5 mM). The results show that increasing quinic acid concentration produces a significant inhibitory effect on the reverse reaction (Fig. 32). It would be interesting in future experiments to compare this with *Cc*HCT.

[00228] The effect of varying the CoA concentration in the reverse reaction was also explored. As expected, increasing the CoA concentration while maintaining 5-CQA levels constant pushes the reverse reaction towards the formation of more caffeoyl-CoA (Fig. 33).

[00229] Thus, caffeoyl-CoA formation depends on the initial concentration of 5-CQA, CoA and quinic acid. It should be noted that the production of caffeoyl-CoA by *Cc*HQT was

initially used for the production of this compound. However, as this reaction reaches equilibrium well below the 100 % conversion rate due to the forward reaction, it is not efficient. The preliminary data on the properties of *Cc*HCT and *Cc*HQT enzymes presented above provides a basis for an understanding of the kinetics of these enzymes.

Formation of diCQA in the reverse reaction

[00230] In reactions containing 5-CQA and CoA catalysed by *Cc*HCT, unknown peaks were observed at later retention times (Fig. 34C). To confirm the identity of these peaks, CGA standards were run, confirming their assignments as 3,4-, 3,5- and 4,5-diCQA. DiCQA formation was observed with *Cc*HCT when 5-CQA and CoA were supplied in a 1:1 molar ratio but not when it was 1:10 (Fig. 34C, D). DiCQAs were not observed when using *Cc*HQT (Fig. 34E, F).

Comparison of the activity of native and mutant HCTs

Comparison of native and mutant HCT in the forward and reverse reactions

[00231] To determine the importance of specific residues on the enzymatic activity of *Cc*HCT, point amino acid mutations were introduced. The activities of the mutant HCTs tested (K, H35A, H153A, H154A, D157A, Y255A, Y252A and R374E) were compared to that of the native *Cc*HCT. All mutants were incubated with 1 mM CoA and 1 mM 5-CQA (reverse reaction), or with 0.2 mM caffeoyl-CoA and 1 mM quinic/ shikimic acid (forward reaction). Fig. 35 shows a summary of results when either 5-CQA and CoA or caffeoyl-CoA and quinic acid were used.

[00232] In the forward reaction, both the native and the K-mutant HCTs used caffeoyl-CoA and quinic acid to form 5-CQA and, to a lesser extent, free caffeic acid. For both proteins there appears to be a complete conversion of the caffeoyl-CoA substrate. When using caffeoyl-CoA and shikimic acid as substrates, the K-mutant HCT catalysed the formation of all three caffeoylshikimic acids produced by native *Cc*HCT (data not shown). In the reverse reaction using 5-CQA and CoA, both the native and K-mutant HCTs produced low levels of caffeoyl-CoA and free caffeic acid, indicating only a partial conversion of 5-CQA to the thioester product.

[00233] In the forward reaction with caffeoyl-CoA and quinic acid, the mutants H35A and Y255A showed a relatively weak catalytic activity, with only a partial conversion of caffeoyl-CoA into 5-CQA, and caffeic acid. With shikimic acid, the H35A and Y255A mutants were also

weakly catalytically active (data not shown). Neither the H35A nor the Y255A mutant HCTs showed any detectable activity in the reverse reaction.

[00234] In the forward reaction, the mutants D157A and R374E only produced caffeic acid in the presence of caffeoyl-CoA and quinic acid. The Y252A mutant produced caffeic acid and a small amount of 5-CQA. In comparison to the H35A and Y255A mutants, these mutants fully transformed the caffeoyl-CoA substrate provided into caffeic acid. In the presence of caffeoyl-CoA and shikimic acid, the D157A mutant HCT exclusively formed caffeic acid (data not shown). The Y252A and R374E mutants were not tested with caffeoyl-CoA and shikimic acid. In the reverse reaction, D157A, Y252A and R374E mutants showed no activity towards 5-CQA and CoA as no peak of caffeoyl-CoA was detected. It is noted however that Y252A did produce caffeic acid. These results suggest that these mutants are not effective in the reverse reaction involving the quinate ester. Most probably, the Y252A mutant is capable of making caffeoyl-CoA, which is subsequently degraded into caffeic acid via a putative lyase activity.

[00235] In accordance with its proposed role in catalysis, the H153A mutant was inactive in the forward reaction involving caffeoyl-CoA and quinic or shikimic acid. As expected, this mutant is also inactive in the reverse direction with 5-CQA and CoA. No caffeic acid was detected. Most interesting are the H154A and H154N/A155L/A156S mutants, which formed 5-CQA and diCQAs from caffeoyl-CoA and quinic acid with a major peak corresponding to the 3,5-diester. The triple mutant also formed a small amount of free caffeic acid. In the reverse direction with 5-CQA and CoA, the two mutants formed a low level of caffeoyl-CoA and a relatively significant amount of diCQAs, again suggesting the importance of the 5-CQA/CoA molar ratio. The major diCQA peak corresponds to the 3,5-diester from which low amounts of the other two peaks, the 3,4- and 4,5-diCQA isomers, were presumably derived by chemical isomerisation. Again, the triple mutant also produced a small amount of free caffeic acid.

Additional analysis of the H154 mutants in the reverse reaction

[00236] The results presented in Fig. 35 suggest that the mutants targeting His154 favour the synthesis of diCQAs when compared to native *Cc*HCT. The two mutants involving H154 were thus further studied by doing a new comparison between the native HCT and the H154A and H154N/A155L/A156S mutants. In the forward reaction, caffeoyl-CoA and quinic or shikimic acid or both were supplied. The results showed that the H154 mutants have comparable

activities to the native enzyme, namely they both continue to show a clear preference for shikimic over quinic acid with caffeoyl-CoA as the second substrate. Caffeic acid formation was observed in the triple mutant only. In the forward reaction with caffeoyl-CoA and shikimic acid, a major peak was detected that is thought to correspond to a caffeoylshikimic acid isomer and two other smaller peaks that are thought to be the two other CSA isomers were seen (data not shown). These results are similar to results obtained with native *Cc*HCT (Fig. 17). The effect of different 5-CQA/CoA molar ratios (1/1 and 1/10) supplied in the reaction was also tested. This experiment confirmed that both mutants again produced diCQAs. Fig. 36 also shows that adding more CoA slightly increased the amount caffeoyl-CoA produced, and that the level of diCQA decreases.

Formation of mixed diesters

To determine if the native and H154 mutant HCTs could form mixed diesters of quinic acid (e.g. caffeoylferuloylquinic acids found in coffee extracts), the enzymes were incubated with 5-CQA/ 5-FQA and CoA/ caffeoyl-/ feruloyl-/ coumaroyl-CoA thioesters. As in the previous experiment, the H154 mutants formed higher levels of these diesters. The results concerning the H154A mutant with 5-CQA and CoA or diverse acyl-CoA thioesters are presented in Fig. 37. The control with no enzyme (Panel A) and all enzymatic reactions showed a partial isomerisation of 5-CQA (RT= 15.9 min) to the 3- and 4-CQA isomers (RT= 7.4 and 14.5 min) after overnight incubation in 0.1 M sodium phosphate pH 6.5. In the presence of 10 mM 5-CQA and 0.5 mM CoA (Panel B), as well as 10 mM 5-CQA and 0.5 mM caffeoyl-CoA (Panel C), three peaks corresponding to 3,4-, 3,5- and 4,5-diCQAs (RT= 41.4, 42.4 and 43.6 min respectively) were formed with native HCT. Interestingly, when 5-CQA and feruloyl-CoA are incubated with H154A mutant, all three diCQAs are synthesised. Three additional peaks formed at RT= 43.9, 45.0 and 45.9 min may correspond to mixed caffeoylferuloylquinic acids. When 5-CQA and coumaroyl-CoA are incubated with the H154A mutant, the three diCQAs are formed (RT= 41.4, 42.4 and 43.6 min), plus two additional peaks at RT= 44.6 and 45.7 min that may correspond to mixed caffeoylcoumaroylquinic acids (Panel E).Similarly, the H154A mutant was incubated with 5-FQA and CoA or diverse acyl-CoA thioesters (Fig. 38). The control with no enzyme (Panel A) and all enzymatic reactions showed a partial isomerisation of 5-FQA (RT= 32.3 min) to the 3- and 4-FQA isomers (RT= 13.7 and 28.2 min) after overnight incubation in 0.1 M sodium

phosphate pH 6.5. Additionally, 4 unknown peaks (RT= 43.7, 44.5, 47.7 and 48.4 min) were observed at later retention times, but these showed a maximum absorbance value at 256 nm, so they were not CGA molecules. Consequently, these peaks are not described in the following enzymatic reactions. With 5-FQA and either CoA or feruloyl-CoA, a new peak appeared at RT= 46.3 min that may correspond to a diferuloylquinic acid (Panels B and D). When 5-FQA and caffeoyl-CoA were incubated, 4 new peaks at RT= 44.2, 45.3, 46.1 and 46.3 min were observed. These were presumed to be the same diFQA (RT= 46.3 min) and mixed caffeoylferuloylquinic acids. When 5-FQA and coumaroyl-CoA were incubated, the same diferuloylquinic acid (RT= 46.3 min) and presumably mixed coumaroylferuloylquinic acids were obtained (RT= 45.4 and 47.3 min).

[00237] In summary, the H154 mutants seem to favour diCQA formation. When feruloyl- or coumaroyl-CoA thioesters were supplied with 5-CQA/ 5-FQA, new peaks with a typical quinate ester absorbance spectrum were detected at late retention times. They were presumed to be new esters of quinic acid. New peaks were observed and putatively attributed to either diferuloylquinic acids (diFQAs) (RT= 45.5, 46.3, and 47.5 min) or mixed diesters, feruloylcaffeoylquinic (RT= 44.2, 45.3, and 46.1 min) and feruloylcoumaroylquinic acids (RT= 45.4, 46.3, and 47.3 min), their identity should to be confirmed by mass spectrometry analysis. These assays show that these enzymes may be involved in the biosynthesis of not only caffeoylquinic, feruloylquinic and coumaroylquinic acids but also mixed diesters that are present in coffee (e.g. caffeoylferuloylquinic acids). The identity of the new products that were formed must be confirmed by comparison with authentic standards or by mass spectrometry analysis.

Production of Chlorogenic or Dactyilfric Acids in vitro Using Combinations of 4CL, HCT and/or HQT

[00238] Reaction mixtures containing 0.5 mM coumaric/ caffeic acid, 0.5/ 10 mM quinic/ shikimic acid, 2.5 mM CoA, 2.5 mM ATP and 5 mM MgCl2 in 0.1 M sodium phosphate pH 6.0 were prepared in a 500 µL final volume. Reactions were started by adding 0.4 µM Nt4CL2 and incubated at 35°C. 75 µL-volume samples were taken at t= 10 min and 5-fold dilutions (824-833) were analysed using HPLC-PDA with the methanol method. A t= 15 min, 0.5 µM HCT/ HQT was added to the remaining 425 µL reaction mixture. Samples (834-863) taken at t= 30

min (15 min after adding HCT/ HQT), t= 105 min and overnight (data not shown for the latter two time points) were also analysed. The experimental setup is shown below in Table 12.

Table 12.

Sample #	Enzyme	Acid	Concentration of acid (mM)	Acceptor	Concentration of acceptor (mM)	Samples taken at t (min)
824	4CL	coumaric	0.5	quinic	10	10
825	4CL	coumaric	0.5	quinic	0.5	10
826	4CL	coumaric	0.5	shikimic	10	10
827	4CL	coumaric	0.5	shikimic	0.5	10
828	4CL	caffeic	0.5	quinic	10	10
829	4CL	caffeic	0.5	quinic	0.5	10
830	4CL	caffeic	0.5	shikimic	10	10
831	4CL	caffeic	0.5	shikimic	0.5	10
832	-	coumaric	0.5	quinic	10	10
833	-	coumaric	0.5	shikimic	10	10
834	4CL + HQT	coumaric	0.5	quinic	10	30
835	4CL + HQT	coumaric	0.5	quinic	0.5	30
836	4CL + HCT	coumaric	0.5	shikimic	10	30
837	4CL + HCT	coumaric	0.5	shikimic	0.5	30
838	4CL + HQT	caffeic	0.5	quinic	10	30
839	4CL + HQT	caffeic	0.5	quinic	0.5	30
840	4CL + HCT	caffeic	0.5	shikimic	10	30
841	4CL + HCT	caffeic	0.5	shikimic	0.5	30
842	HQT	coumaric	0.5	quinic	10	30
843	HCT	coumaric	0.5	shikimic	10	30

[00239] Results are shown in Fig. 39. Panel A shows results of a reaction with no enzyme and 0.5 mM coumaric acid as substrate. Panel B shows results of a reaction with 4CL alone and 0.5mM coumaric acid as substrate. As can be seen, in the presence of coumaric acid, CoA, ATP and Mg2+, 4CL forms coumaroyl-CoA. Panel C shows the results of consecutive reactions with 4CL and HQT with coumaric (0.5 mM) and quinic (0.5/ 10 mM) acids as substrates. Here, HQT converts coumaroyl-CoA produced by 4CL to a coumaroylquinic acid. The conversion is more efficient with 10mM (samples 834, 844, 854) than 0.5mM (samples 835, 845, 855) quinic acid.. Panel D shows consecutive reactions with 4CL and HCT with coumaric (0.5 mM) and shikimic (0.5/ 10 mM) acids as substrates. In this reaction, HCT converts coumaroyl-CoA produced by 4CL to a coumaroylshikimic acid after 30 min. The conversion is more efficient with 10mM (samples 836, 846, 856) than 0.5mM (samples 837, 847, 857) quinic acid, as a significant amount of coumaroyl-CoA remains in the latter.

[00240] Panel E shows results of an initial reaction with 4CL alone and 0.5mM caffeic acid as substrate. As can be seen, caffeic acid is converted to caffeoyl-CoA by 4CL in the presence of CoA, ATP and Mg2+. Panel F shows results of consecutive reactions with 4CL and HQT with caffeic (0.5 mM) and quinic (0.5/ 10 mM) acids as substrates. Here, HQT converts caffeoyl-CoA (produced by 4CL) to 5-CQA. The conversion is more efficient with 10 mM than 0.5 mM quinic acid. The conversion is complete with 10 mM quinic acid and almost no caffeoyl-CoA remains after 15 min (samples 838, 848, 858). Caffeic acid is still present in sample 839, so 4CL has not converted all the precursor to caffeoyl-CoA yet. Panel G shows the results of consecutive reactions with 4CL and HCT with caffeic (0.5 mM) and shikimic (0.5/ 10 mM) acids as substrates. In this reaction, HCT converts caffeoyl-CoA produced by 4CL to a caffeoylshikimic acid after 10min. The conversion is more efficient with 10 mM than 0.5 mM shikimic acid as some caffeoyl-CoA remains in the latter.

[00241] The experimental results set forth above demonstrate that chlorogenic acids or dactylifric acids can be produced in a single *in-vitro* reaction. The chlorogenic or dactyfrilic acids are generated using either caffeic acid or coumaric acid and either quinic acid or shikimic acid, with the inclusion of recombinant 4CL and either recombinant HCT or recombinant HQT and the appropriate cofactors and buffer conditions. In the experiements described above, the reactions were carried out as partially consecutive steps, *i.e.,* 4CL was added before HCT or HQT. However, it is expected that the same or similar results would be observed if the enzymes were added together. This makes feasible the performance of such reactions *in situ*, in recombinant microbial or plant cells co-expressing the enzymes. The data shown above also suggest that such co-reactions may "push" the reaction towards the production of more chlorogenic acids or dactylifric acids, in particular when the quinic acid and shikimic acids are in high concentrations.

III. Use of Synthesized Chlorogenic Acid Species in the Production of a Coffee Product

Hypothetical Production of a Coffee Product

[00242] A immobilised enzyme system will be constructed to produce commercially useful quantities of CGAs and DAs. The enzymes will be cloned CcHCT and CcHQT which are

over-expressed in food-grade lactic acid bacteria, and purified to homogeneity (as seen on SDS gel electrophoresis) with a single-step column separation.

[00243] The enzymes will be used to catalyze the production of CGA and DA species. Preferably caffeic acid is activated to the -CoA thioester using a 4CL enzyme in a separate preparatory step that proceeds nearly completely to product formation. The caffeoyl-CoA will be used as the substrate and is to be combined with quinic acid and/or a combination of quinic and shikimic acid in a semi-continuous process to yield caffeoylquinic and shikimic esters, as well as diesters. The conditions are optimized for the production of 3,4, caffeoylquinic acid as a major product. The product of the immobilised enzyme system is a food-grade mixture of CGA and DA species that includes some remaining caffeoyl-CoA.

[00244] A food product in the form of a soluble coffee product is to be manufactured according to procedures described above. Prior to completing the product and packaging the product for consumers, a mixture of exogenous chlorogenic acids and/or dactylifric acid species will be added to the coffee product to improve the flavour profile and aroma. The mixture is preferably enriched in 3,4 dicaffeoylquinic acid, and relatively deficient in 5-feruloylquinic acid. The addition of the exogenous mixture will provide several advantages to the soluble coffee product. The flavour of the product will be improved. Consumers will note that there is slight but desirable bitter taste, and a pleasant roasted aroma and flavour. Consumers will also note a degree of astringency that is not unpleasant or overpowering.

[00245] In the specification, there have been disclosed typical preferred embodiments of the invention. Although specific terms are employed, they are used in a generic and descriptive sense only and not for purposes of limiting the claims. The scope of the invention is set forth in the claims. In light of the above teachings, many modifications and variations of the invention are possible and may be apparent to the skilled artisan. It is therefore to be understood that within the scope of the appended claims the invention may be practiced otherwise than as specifically described.

REFERENCES

Abdullah, Y., B. Schneider and M. Petersen (2008). "Occurrence of rosmarinic acid, chlorogenic acid and rutin in Marantaceae species." Phytochemistry Letters **1**(4): 199-203.

Abdulrazzak, N., B. Pollet, J. Ehlting, K. Larsen, *et al.* (2006). "A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of nonredundant meta-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth." Plant Physiol **140**(1): 30-48.

Aerts, R. J. and T. W. Baumann (1994). "Distribution and utilization of chlorogenic acid in Coffea seedlings." Journal of Experimental Botany **45**(273): 497-503.

Arnold, K., L. Bordoli, J. Kopp and T. Schwede (2006). "The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling." Bioinformatics **22**(2): 195-201.

Ashihara, H., H. Sano and A. Crozier (2008). "Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering." Phytochemistry **69**: 841–856.

Baldi, L., D. L. Hacker, M. Adam and F. M. Wurm (2007). "Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives." Biotechnology Letters **29**(5): 677-684.

Baneyx, F. (1999). "Recombinant protein expression in Escherichia coli." Current Opinion in Biotechnology **10**(5): 411-421.

Baucher, M., C. Halpin, M. Petit-Conil and W. Boerjan (2003). "Lignin: genetic engineering and impact on pulping." Crit Rev Biochem Mol Biol **38**(4): 305-350.

Becker-Andre, M., P. Schulze-Lefert and K. Hahlbrock (1991). "Structural comparison, modes of expression and putative cis-acting elements of the two 4-coumarate CoA ligase genes in potato." Journal of Biological Chemistry **266**(13): 8551-8559.

Berger, A., J. Meinhard and M. Petersen (2006). "Rosmarinic acid synthase is a new member of the superfamily of BAHD acyltransferases." Planta **224**(6): 1503-1510.

Berman, H. M., T. Battistuz, T. N. Bhat, W. F. Bluhm, *et al.* (2002). "The Protein Data Bank." Acta Crystallographica Section D-Biological Crystallography **58**: 899-907.

Bertrand, B., B. Guyot, F. Anthony and P. Lashermes (2003). "Impact of the coffea canephora gene introgression on beverage quality of C-arabica." Theoretical and Applied Genetics **107**(3): 387-394.

Besseau, S., L. Hoffmann, P. Geoffroy, C. Lapierre, *et al.* (2007). "Flavonoid accumulation in Arabidopsis repressed in lignin synthesis affects auxin transport and plant growth." Plant Cell **19**(1): 148-162.

Beuerle, T. and E. Pichersky (2002). "Enzymatic synthesis and purification of aromatic coenzyme a esters." Anal Biochem **302**(2): 305-312.

Bisswanger, H. (2002). "Enzyme Kinetics: Principles and Methods." John Wiley And Sons Ltd.

Boerjan, W., J. Ralph and M. Baucher (2003). "Lignin biosynthesis." Annu Rev Plant Biol **54**: 519-546.

Branchini, B. R., M. H. Murtiashaw, R. A. Magyar and S. M. Anderson (2000). "The role of lysine 529, a conserved residue of the acyl-adenylate-forming enzyme superfamily, in firefly luciferase." Biochemistry **39**(18): 5433-5440.

Buglino, J., K. C. Onwueme, J. A. Ferreras, L. E. Quadri, *et al.* (2004). "Crystal structure of PapA5, a phthiocerol dimycocerosyl transferase from Mycobacterium tuberculosis." J. Biol. Chem. **279**(29): 30634-30642.

Burchard, P., W. Bilger and G. Weissenböck (2000). "Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements." Plant Cell Env. **23**(12): 1373-1380.

Calabrese, J. C., D. B. Jordan, A. Boodhoo, S. Sariaslani, *et al.* (2004). "Crystal structure of phenylalanine ammonia lyase: Multiple helix dipoles implicated in catalysis." Biochemistry **43**(36): 11403-11416.

Caldwell, M. M., R. Robberecht and S. D. Flint (1983). "Internal filters - Prospects for UV-acclimation in higher plants." Physiologia Plantarum **58**(3): 445-450.

Campa, C., S. Doulbeau, S. Dussert, S. Hamon, *et al.* "Discontinuous distribution of caffeine content among wild Coffea species." Food Chemistry.

Campbell, M. M. and R. R. Sederoff (1996). "Variation in Lignin Content and Composition (Mechanisms of Control and Implications for the Genetic Improvement of Plants)." Plant Physiol **110**(1): 3-13.

Carter, C. W. and S. B. Xiang (1997). Phase improvement using conditional probability methods: Maximum entropy solvent flattening and phase permutation. Macromolecular Crystallography, Pt B. **277:** 79-109.

Cerovic, Z. G., A. Ounis, A. Cartelat, G. Latouche, *et al.* (2002). "The use of chlorophyll fluorescence excitation spectra for the non-destructive in situ assessment of UV-absorbing compounds in leaves." Plant Cell and Environment **25**(12): 1663-1676.

Chapple, C. C., T. Vogt, B. E. Ellis and C. R. Somerville (1992). "An Arabidopsis mutant defective in the general phenylpropanoid pathway." Plant Cell **4**(11): 1413-1424.

Chen, F. and R. A. Dixon (2007). "Lignin modification improves fermentable sugar yields for biofuel production." Nat Biotechnol **25**(7): 759-761.

Chen, V. B., W. B. Arendall, J. J. Headd, D. A. Keedy, *et al.* (2010). "MolProbity: all-atom structure validation for macromolecular crystallography." Acta Crystallographica Section D-Biological Crystallography **66**: 12-21.

Cheng (2007). "Plant Terpenoids: Biosynthesis and Ecological Functions." Journal of Integrative Plant Biology **49**(2): 179–186.

Cieslik, M. and Z. S. Derewenda (2009). "The role of entropy and polarity in intermolecular contacts in protein crystals." Acta Crystallographica Section D-Biological Crystallography **65**: 500-509.

Cle, C., L. M. Hill, R. Niggeweg, C. R. Martin, *et al.* (2008). "Modulation of chlorogenic acid biosynthesis in Solanum lycopersicum; consequences for phenolic accumulation and UV-tolerance." Phytochemistry **69**(11): 2149-2156.

Clifford, M. N. (1979). "Chlorogenic acids - Their complex nature and routine determination in coffee beans." Food Chemistry **4**(1): 63-71.

Clifford, M. N. (1985). "Chlorogenic acids. In: R. J. Clarke & R. Macrae (Eds) Coffee, Elsevier Applied Science, London." Chemistry **1**: 153-202.

Clifford, M. N. (1999). "Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden." Journal of the Science of Food and Agriculture **79**(3): 362-372.

Clifford, M. N. (2000). "Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism." Journal of the Science of Food and Agriculture **80**(7): 1033-1043.

Clifford, M. N. (2004). "Diet-derived Phenols in plasma and tissues and their implications for health." Planta Medica **70**(12): 1103-1114.

Clifford, M. N., S. Marks, S. Knight and N. Kuhnert (2006). "Characterization by LC-MS(n) of four new classes of p-coumaric acid-containing diacyl chlorogenic acids in green coffee beans." J Agric Food Chem **54**(12): 4095-4101.

Comino, C., A. Hehn, A. Moglia, B. Menin, *et al.* (2009). "The isolation and mapping of a novel hydroxycinnamoyltransferase in the globe artichoke chlorogenic acid pathway." BMC Plant Biol **9**: 30.

Comino, C., S. Lanteri, E. Portis, A. Acquadro, *et al.* (2007). "Isolation and functional characterization of a cDNA coding a hydroxycinnamoyltransferase involved in phenylpropanoid biosynthesis in Cynara cardunculus L." BMC Plant Biol **7**: 14.

Conti, E., N. P. Franks and P. Brick (1996). "Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes." Structure **4**(3): 287-298.

Conti, E., T. Stachelhaus, M. A. Marahiel and P. Brick (1997). "Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S." Embo Journal **16**(14): 4174-4183.

Costa, M. A., D. L. Bedgar, S. G. A. Moinuddin, K.-W. Kim, *et al.* (2005). "Characterization in vitro and in vivo of the putative multigene 4-coumarate:CoA ligase network in Arabidopsis: syringyl lignin and sinapate/sinapyl alcohol derivative formation." Phytochemistry **66**(17): 2072-2091.

Cukovic, D., J. Ehlting, J. A. VanZiffle and C. J. Douglas (2001). "Structure and evolution of 4-coumarate : coenzyme A ligase (4CL) gene families." Biological Chemistry **382**(4): 645-654.

D'Auria, J. C. (2006). "Acyltransferases in plants: a good time to be BAHD." Curr. Opin. Plant Biol. **9**(3): 331-340.

Davis, I. W., A. Leaver-Fay, V. B. Chen, J. N. Block, *et al.* (2007). "MolProbity: all-atom contacts and structure validation for proteins and nucleic acids." Nucleic Acids Res **35**(Web Server issue): W375-383.

Dawidowicz, A. and R. Typek (2010). "Thermal stability of 5-O-caffeoylquinic acid in aqueous solutions at different heating conditions." J Agric Food Chem **58**(24): 12578-12584.

De Castro, R. D. and P. Marraccini (2006). "Cytology, biochemistry and molecular changes during coffee fruit development." Brazilian Journal of Plant Physiology **18**: 175-199.

de Paulis, T., P. Commers, A. Farah, J. L. Zhao, *et al.* (2004). "4-caffeoyl-1,5-quinide in roasted coffee inhibits H-3 naloxone binding and reverses anti-nociceptive effects of morphine in mice." Psychopharmacology **176**(2): 146-153.

Derewenda, Z. S. and P. G. Vekilov (2006). "Entropy and surface engineering in protein crystallization." Acta Crystallographica Section D-Biological Crystallography **62**: 116-124.

Dixon, R. A. (2005). "Engineering of plant natural product pathways." Current Opinion in Plant Biology **8**(3): 329-336.

Dixon, R. A., C. J. Lamb, S. Masoud, V. J. Sewalt, *et al.* (1996). "Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses--a review." Gene **179**(1): 61-71.

Dixon, R. A. and N. L. Paiva (1995). "Stress-induced phenylpropanoid metabolism." Plant Cell **7**(7): 1085-1097.

Douglas, C. J. (1996). "Phenylpropanoid metabolism and lignin biosynthesis: From weeds to trees." Trends in Plant Science **1**(6): 171-178.

Drewnowski, A. (1997). "Taste preferences and food intake." Annual Review of Nutrition **17**: 237-253.

Dudareva, N., E. Pichersky and J. Gershenzon (2004). "Biochemistry of plant volatiles." Plant Physiology **135**(4): 1893-1902.

Ehlting, J., D. Buttner, Q. Wang, C. J. Douglas, *et al.* (1999). "Three 4-coumarate : coenzyme A ligases in Arabidopsis thaliana represent two evolutionarily divergent classes in angiosperms." Plant Journal **19**(1): 9-20.

Ehlting, J., N. J. Provart and D. Werck-Reichhart (2006). "Functional annotation of the Arabidopsis P450 superfamily based on large-scale co-expression analysis." Biochemical Society Transactions **34**: 1192-1198.

Emsley, P. and K. Cowtan (2004). "Coot: model-building tools for molecular graphics." Acta Crystallogr. D Biol. Crystallogr. **60**(Pt 12 Pt 1): 2126-2132.

Ericsson, U. B., B. M. Hallberg, G. T. Detitta, N. Dekker, *et al.* (2006). "Thermofluor-based high-throughput stability optimization of proteins for structural studies." Anal. Biochem. **357**(2): 289-298.

Esposito, D. and D. Chatterjee (2006). "Enhancement of soluble protein expression through the use of fusion tags." Curr Opin Biotechnol **17**: 353-358.

Evans, P. and A. McCoy (2008). "An introduction to molecular replacement." Acta Crystallographica Section D-Biological Crystallography **64**: 1-10.

Farah, A., T. de Paulis, L. C. Trugo and P. R. Martin (2005). "Effect of roasting on the formation of chlorogenic acid lactones in coffee." J Agric Food Chem **53**(5): 1505-1513.

Farah, A., M. C. Monteiro, V. Calado, A. S. Franca, *et al.* (2006). "Correlation between cup quality and chemical attributes of Brazilian coffee." Food Chemistry **98**(2): 373-380.

Ferrer, J. L., M. B. Austin, C. Stewart, Jr. and J. P. Noel (2008). "Structure and function of enzymes involved in the biosynthesis of phenylpropanoids." Plant Physiol Biochem **46**(3): 356-370.

Ferrer, J. L., J. M. Jez, M. E. Bowman, R. A. Dixon, *et al.* (1999). "Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis." Nature Structural Biology **6**(8): 775-784.

Finley, J. W. (2005). "Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols and selenocompounds." Ann Bot **95**(7): 1075-1096.

Flot, D., T. Mairs, T. Giraud, M. Guijarro, *et al.* (2010). "The ID23-2 structural biology microfocus beamline at the ESRF." J. Synchrotron Radiat. **17**(1): 107-118.

Foley, S., S. Navaratnam, D. J. McGarvey, E. J. Land, *et al.* (1999). "Singlet oxygen quenching and the redox properties of hydroxycinnamic acids." Free Radical Biology and Medicine **26**(9-10): 1202-1208.

Frank, O., G. Zehentbauer and T. Hofmann (2006). "Bioresponse-guided decomposition of roast coffee beverage and identification of key bitter taste compounds." European Food Research and Technology **222**(5-6): 492-508.

Franke, R., J. M. Humphreys, M. R. Hemm, J. W. Denault, *et al.* (2002). "The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism." Plant Journal **30**(1): 33-45.

Freyer, M. W. and E. A. Lewis (2008). Isothermal titration calorimetry: Experimental design, data analysis, and probing Macromolecule/Ligand binding and kinetic interactions. Biophysical Tools for Biologists: Vol 1 in Vitro Techniques. **84:** 79-113.

Fulda, M., E. Heinz and F. P. Wolter (1994). "The fadD gene of Escherichia coli K12 is located close to rnd at 39.6 min of the chromosomal map and is a new member of the AMP-binding protein family." Mol Gen Genet **242**(3): 241-249.

Garman, E. F. and S. M. McSweeney (2007). "Progress in research into radiation damage in cryo-cooled macromolecular crystals." Journal of Synchrotron Radiation **14**: 1-3.

Garvey, G. S., S. P. McCormick, N. J. Alexander and I. Rayment (2009). "Structural and functional characterization of TRI3 trichothecene 15-O-acetyltransferase from Fusarium sporotrichioides." Protein Sci. **18**(4): 747-761.

Garvey, G. S., S. P. McCormick and I. Rayment (2008). "Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium

sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight." J. Biol. Chem. **283**(3): 1660-1669.

Gershenzon, J. and N. Dudareva (2007). "The function of terpene natural products in the natural world." Nature Chemical Biology **3**(7): 408-414.

Goodsell, D. S., G. M. Morris and A. J. Olson (1996). "Automated docking of flexible ligands: applications of AutoDock." J Mol Recognit **9**(1): 1-5.

Gouet, P., E. Courcelle, D. I. Stuart and F. Metoz (1999). "ESPript: analysis of multiple sequence alignments in PostScript." Bioinformatics **15**(4): 305-308.

Gouet, P., X. Robert and E. Courcelle (2003). "ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins." Nucleic Acids Res **31**(13): 3320-3323.

Goupy, P. M., P. J. A. Varoquaux, J. J. Nicolas and J. J. Macheix (1990). "Identification and localization of hydroxycinnamoyl and flavonol derivatives from endive (Cichorium endivia L. cv geante maraichere) leaves." Journal of Agricultural and Food Chemistry **38**(12): 2116-2121.

Grienenberger, E., S. Besseau, P. Geoffroy, D. Debayle, *et al.* (2009). "A BAHD acyltransferase is expressed in the tapetum of Arabidopsis anthers and is involved in the synthesis of hydroxycinnamoyl spermidines." Plant Journal **58**(2): 246-259.

Griffiths, D. W. and H. Bain (1997). "Photo-induced changes in the concentrations of individual chlorogenic acid isomers in potato (Solanum tuberosum) tubers and their complexation with ferric ions." Potato Research **40**(3): 307-315.

Griffiths, D. W., H. Bain and M. F. B. Dale (1992). "Development of a rapid colourimetric method for the determination of chlorogenic acid in freeze-dried potato tubers." Journal of the Science of Food and Agriculture **58**(1): 41-48.

Gulick, A. M. (2009). "Conformational Dynamics in the Acyl-CoA Synthetases, Adenylation Domains of Non-ribosomal Peptide Synthetases, and Firefly Luciferase." Acs Chemical Biology **4**(10): 811-827.

Hahlbrock, K. and D. Scheel (1989). "Physiology and molecular biology of phenylpropanoid metabolism." Annual Review of Plant Physiology and Plant Molecular Biology **40**: 347-369.

Hamberger, B. and K. Hahlbrock (2004). "The 4-coumarate:CoA ligase gene family in Arabidopsis thaliana comprises one rare, sinapate-activating and three commonly occurring isoenzymes." Proc Natl Acad Sci U S A **101**(7): 2209-2214.

Harborne, J. B., C. A. Williams, J. Greenham and P. Moyna (1974). "Distribution of charged flavones and caffeoylshikimic acid in Palmae." Phytochemistry **13**(8): 1557-1559.

Hartmann, T. (2007). "From waste products to ecochemicals: Fifty years research of plant secondary metabolism." Phytochemistry **68**(22-24): 2831-2846.

Hendlich, M., F. Rippmann and G. Barnickel (1997). "LIGSITE: Automatic and efficient detection of potential small molecule-binding sites in proteins." Journal of Molecular Graphics & Modelling **15**(6): 359-+.

Hendrickson, W. A., A. Pahler, J. L. Smith, Y. Satow, *et al.* (1989). "Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation." Proceedings of the National Academy of Sciences of the United States of America **86**(7): 2190-2194.

Herrmann, K. M. (1995). "The shikimate pathway - Early steps in the biosynthesis of aromatic compounds." Plant Cell 7(7): 907-919.

Herrmann, K. M. and L. M. Weaver (1999). "The shikimate pathway." Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**: 473–503.

Heyda, J., P. Mason and P. Jungwirth (2010). "Attractive interactions between side chains of histidine-histidine and histidine-arginine-based cationic dipeptides in water." J Phys Chem B **114**(26): 8744-8749.

Hoffmann, L., S. Besseau, P. Geoffroy, C. Ritzenthaler, *et al.* (2004). "Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis." Plant Cell **16**(6): 1446-1465.

Hoffmann, L., S. Besseau, P. Geoffroy, C. Ritzenthaler, *et al.* (2005). "Acyltransferase-catalysed p-coumarate ester formation is a committed step of lignin biosynthesis." Plant Biosystems **139**(1): 50-53.

Hoffmann, L., S. Maury, F. Martz, P. Geoffroy, *et al.* (2003). "Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism." J Biol Chem **278**(1): 95-103.

Holm, L. and P. Rosenstrom (2010). "Dali server: conservation mapping in 3D." Nucleic Acids Research **38**: W545-W549.

Horswill, A. R. and J. C. Escalante-Semerena (2002). "Characterization of the propionyl-CoA synthetase (PrpE) enzyme of Salmonella enterica: Residue Lys592 is required for propionyl-AMP synthesis." Biochemistry **41**(7): 2379-2387.

Hu, W. J., A. Kawaoka, C. J. Tsai, J. H. Lung, *et al.* (1998). "Compartmentalized expression of two structurally and functionally distinct 4-coumarate : CoA ligase genes in aspen (Populus tremuloides)." Proceedings of the National Academy of Sciences of the United States of America **95**(9): 5407-5412.

Hunt, I. (2005). "From gene to protein: a review of new and enabling technologies for multiparallel protein expression." Protein Expr Purif **40**: 1-22.

Ilari, A. and C. Savino (2008). "Protein structure determination by X-ray crystallography." Bioinformatics **452**(3).

IUPAC (1976). "Nomenclature of cyclitols." Biochem. J. **153**: 23-31.

Iwashina, T. (2003). "Flavonoid function and activity to plants and other organisms." Biol Sci Space **17**(1): 24-44.

Jaiswal, R. (2010). "Profile and Characterization of the Chlorogenic Acids in Green Robusta Coffee Beans by LC-MSn: Identification of Seven New Classes of Compounds." J. Agric. Food Chem. **58**: 8722–8737.

Jiang, D. S. and D. G. Peterson (2010). "Role of hydroxycinnamic acids in food flavour: a brief overview." Phytochemistry Reviews **9**(1): 187-193.

Jogl, G. and L. Tong (2003). "Crystal structure of carnitine acetyltransferase and implications for the catalytic mechanism and fatty acid transport." Cell **112**(1): 113-122.

Johnston, K. L., M. N. Clifford and L. M. Morgan (2003). "Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine." Am J Clin Nutr **78**(4): 728-733.

Kabsch, W. (2010). "XDS." Acta Crystallogr. D Biol. Crystallogr. **66**(2): 125-132.

Kahnt, G. (1967). "Trans-cis-equilibrium of hydroxycinnamic acids during irradiation of aqueous solutions at different pH." Phytochemistry **6**(5): 755-&.

Kane, J. F. (1995). "Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli." Curr Opin Biotechnol **6**(5): 494-500.

Kleywegt, G. J. (1996). "Use of non-crystallographic symmetry in protein structure refinement." Acta Crystallographica Section D-Biological Crystallography **52**: 842-857.

Kochan, G., E. S. Pilka, F. von Delft, U. Oppermann, *et al.* (2009). "Structural Snapshots for the Conformation-dependent Catalysis by Human Medium-chain Acyl-coenzyme A Synthetase ACSM2A." Journal of Molecular Biology **388**(5): 997-1008.

Koshiro, Y., M. C. Jackson, R. Katahira, M. L. Wang, *et al.* (2007). "Biosynthesis of chlorogenic acids in growing and ripening fruits of Coffea arabica and Coffea canephora plants." Zeitschrift Fur Naturforschung Section C-a Journal of Biosciences **62**(9-10): 731-742.

Kost, T. A. and J. P. Condreay (1999). "Recombinant baculoviruses as expression vectors for insect and mammalian cells." Current Opinion in Biotechnology **10**(5): 428-433.

Krissinel, E. and K. Henrick (2007). "Inference of macromolecular assemblies from crystalline state." Journal of Molecular Biology **372**(3): 774-797.

Ku, H. M., T. Vision, J. P. Liu and S. D. Tanksley (2000). "Comparing sequenced segments of the tomato and Arabidopsis genomes: Large-scale duplication followed by selective gene loss creates a network of synteny." Proceedings of the National Academy of Sciences of the United States of America **97**(16): 9121-9126.

Ky, C. L., J. Louarn, S. Dussert, B. Guyot, *et al.* (2001). "Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild Coffea arabica L. and C-canephora P. accessions." Food Chemistry **75**(2): 223-230.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, *et al.* (2007). "Clustal W and Clustal X version 2.0." Bioinformatics **23**(21): 2947-2948.

Lattanzio, V., P. A. Kroon, S. Quideau and D. Treutter (2008). Plant Phenolics - Secondary Metabolites with Diverse Functions. Recent Advances in Polyphenol Research, Vol 1. F. Daayf and V. Lattanzio. Oxford, Blackwell Publishing. **1:** 1-35.

Lee, D. and C. J. Douglas (1996). "Two divergent members of a tobacco 4-coumarate:coenzyme A ligase (4CL) gene family - cDNA structure, gene inheritance and expression, and properties of recombinant proteins." Plant Physiology **112**(1): 193-205.

Leloup, V., A. Louvrier and R. Liardon (1995). "Degradation mechanisms of chlorogenic acids during roasting." Proc. 16th Int. Sci. Coll. Coffee (Kyoto) ASIC, Paris: 192-198.

Lepelley, M., G. Cheminade, J. G. McCarthy, V. Pétiard, *et al.* (2007). Polynucleotides encoding phenylpropanoid and flavonoid biosynthetic pathway enzymes in coffee.

Lepelley, M., G. Cheminade, N. Tremillon, A. Simkin, *et al.* (2007). "Chlorogenic acid synthesis in coffee: An analysis of CGA content and real-time RT-PCR expression of HCT, HQT, C3H1, and CCoAOMT1 genes during grain development in C. canephora." Plant Sci. **172**(5): 978-996.

Leslie, J. R. (1988). "Structure of Chloramphenicol Acetyltransferase at 1.75- angstrom Resolution." Proc Natl Acad Sci U S A.

Li, M., Z. Su and J. Janson (2004). "In vitro protein refolding by chromatography procedures." Protein Expr Purif **33**: 1-10.

Liang, Y. (2008). "Applications of isothermal titration calorimetry in protein science." Acta Biochimica Et Biophysica Sinica **40**(7): 565-576.

Lin, C., L. A. Mueller, J. Mc Carthy, D. Crouzillat, *et al.* (2005). "Coffee and tomato share common gene repertoires as revealed by deep sequencing of seed and cherry transcripts." Theor Appl Genet **112**(1): 114-130.

Lindermayr, C., J. Fliegmann and J. Ebel (2003). "Deletion of a single amino acid residue from different 4-coumarate : CoA Ligases from soybean results in the generation of new substrate specificities." Journal of Biological Chemistry **278**(5): 2781-2786.

Lindermayr, C., B. Mollers, J. Fliegmann, A. Uhlmann, *et al.* (2002). "Divergent members of a soybean (Glycine max L.) 4-coumarate : coenzyme A ligase gene family - Primary structures, catalytic properties, and differential expression." European Journal of Biochemistry **269**(4): 1304-1315.

Long, F., A. A. Vagin, P. Young and G. N. Murshudov (2008). "BALBES: a molecular-replacement pipeline." Acta Crystallographica Section D-Biological Crystallography **64**: 125-132.

Lowe (2009). "The great acetonitrile shortage."

Luo, J., C. Fuell, A. Parr, L. Hill, *et al.* (2009). "A Novel Polyamine Acyltransferase Responsible for the Accumulation of Spermidine Conjugates in Arabidopsis Seed." Plant Cell **21**(1): 318-333.

Ma, X., J. Koepke, A. Bayer, V. Linhard, *et al.* (2004). "Vinorine synthase from Rauvolfia: the first example of crystallization and preliminary X-ray diffraction analysis of an enzyme of the BAHD superfamily." Biochim Biophys Acta **1701**(1-2): 129-132.

Ma, X., J. Koepke, S. Panjikar, G. Fritzsch, *et al.* (2005). "Crystal structure of vinorine synthase, the first representative of the BAHD superfamily." J. Biol. Chem. **280**(14): 13576-13583.

Mahesh, V., R. Million-Rousseau, P. Ullmann, N. Chabrillange, *et al.* (2007). "Functional characterization of two p-coumaroyl ester 3'-hydroxylase genes from coffee tree: evidence of a candidate for chlorogenic acid biosynthesis." Plant Mol Biol **64**(1-2): 145-159.

Maier, V. P., D. M. Metzler and A. F. Huber (1964). "3-3-Caffeoylshikimic acid (dactylifric acid) + its isomers a new class of enzymic browning substrates." Biochemical and Biophysical Research Communications **14**(2): 124-&.

Malakhov, M. P., M. R. Mattern, O. A. Malakhova, M. Drinker, *et al.* (2004). "SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins." J Struct Funct Genomics **5**(1-2): 75-86.

Malissard, M., S. Zeng and E. G. Berger (1999). "The yeast expression system for recombinant glycosyltransferases." Glycoconjugate Journal **16**(2): 125-139.

Matsuda, F., K. Morino, R. Ano, M. Kuzawa, *et al.* (2005). "Metabolic flux analysis of the phenylpropanoid pathway in elicitor-treated potato tuber tissue." Plant and Cell Physiology **46**(3): 454-466.

McCarthy, A. A., S. Brockhauser, D. Nurizzo, P. Theveneau, *et al.* (2009). "A decade of user operation on the macromolecular crystallography MAD beamline ID14-4 at the ESRF." J Synchrotron Radiat **16**(Pt 6): 803-812.

McCarty, M. F. (2005). "A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk." Medical Hypotheses **64**(4): 848-853.

McCoy, A. J., R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, *et al.* (2007). "Phaser crystallographic software." J. Appl. Crystallogr. **40**(Pt 4): 658-674.

McDougall, B., P. J. King, B. W. Wu, Z. Hostomsky, *et al.* (1998). "Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase." Antimicrobial Agents and Chemotherapy **42**(1): 140-146.

McPherson, A. (1982). Wiley.

Menin, B., C. Comino, A. Moglia, Y. Dolzhenko, *et al.* (2010). "Identification and mapping of genes related to caffeoylquinic acid synthesis in *Cynara cardunculus* L." Plant Sci. **179**: 338-347.

Meurisse, R., R. Brasseur and A. Thomas (2003). "Aromatic side-chain interactions in proteins. Near- and far-sequence His-X pairs." Biochim Biophys Acta **1649**: 85-96.

Moglia, A., C. Comino, L. S., R. de Vos, *et al.* (2010). "Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast. ." Metab Eng. **12**(3): 223-232.

Mondolot, L., P. La Fisca, B. Buatois, E. Talansier, *et al.* (2006). "Evolution in caffeoylquinic acid content and histolocalization during Coffea canephora leaf development." Annals of Botany **98**(1): 33-40.

Moriguchi, T., R. J. A. Villegas, T. Kondo and M. Kojima (1988). "Isolation of 1-O-trans-para-coumaroyl-beta-deuterium-glucopyranose from sweet-potato roots and examination of its role in chlorogenic acids biosynthesis." Plant and Cell Physiology **29**(7): 1221-1226.

Murata, M., H. Okada and S. Homma (1995). "Hydroxycinnamic acid derivatives and p-coumaroyl-(L)-tryptophan, a novel hydroxycinnamic acid derivative, from coffee beans." Bioscience Biotechnology and Biochemistry **59**(10): 1887-1890.

Murata, M., M. Sugiura, Y. Sonokawa, T. Shimamura, *et al.* (2002). "Properties of chlorogenic acid quinone: Relationship between browning and the formation of hydrogen peroxide from a quinone solution." Bioscience Biotechnology and Biochemistry **66**(12): 2525-2530.

Murshudov, G. N., A. A. Vagin and E. J. Dodson (1997). "Refinement of macromolecular structures by the maximum-likelihood method." Acta Crystallogr D Biol Crystallogr **53**(Pt 3): 240-255.

Nair, R. B., Q. Xia, C. J. Kartha, E. Kurylo, *et al.* (2002). "Arabidopsis CYP98A3 mediating aromatic 3-hydroxylation. Developmental regulation of the gene, and expression in yeast." Plant Physiology **130**(1): 210-220.

Nakatsu, T., S. Ichiyama, J. Hiratake, A. Saldanha, *et al.* (2006). "Structural basis for the spectral difference in luciferase bioluminescence." Nature **440**(7082): 372-376.

Nardini, M., E. Cirillo, F. Natella and C. Scaccini (2002). "Absorption of phenolic acids in humans after coffee consumption." J Agric Food Chem **50**(20): 5735-5741.

Natella, F., M. Nardini, F. Belelli and C. Scaccini (2007). "Coffee drinking induces incorporation of phenolic acids into LDL and increases the resistance of LDL to ex vivo oxidation in humans." American Journal of Clinical Nutrition **86**(3): 604-609.

Niggeweg, R., A. J. Michael and C. Martin (2004). "Engineering plants with increased levels of the antioxidant chlorogenic acid." Nat. Biotechnol. **22**(6): 746-754.

Ohiokpehai, O., G. Brumen and M. N. Clifford (1983). "The chlorogenic acids content of some peculiar green coffee beans and the implications for beverage quality." 10th Int. Colloq. Chem. Coffee (ASIC): 177-186.

Pantoliano, M. W., E. C. Petrella, J. D. Kwasnoski, V. S. Lobanov, *et al.* (2001). "High-density miniaturized thermal shift assays as a general strategy for drug discovery." J Biomol Screen **6**(6): 429-440.

Petersen, M., Y. Abdullah, J. Benner, D. Eberle, *et al.* (2009). "Evolution of rosmarinic acid biosynthesis." Phytochemistry **70**(15-16): 1663-1679.

Petersen, M. and M. S. Simmonds (2003). "Rosmarinic acid." Phytochemistry **62**(2): 121-125.

Pichersky, E. and J. Gershenzon (2002). "The formation and function of plant volatiles: perfumes for pollinator attraction and defense." Current Opinion in Plant Biology **5**(3): 237-243.

Raes, J., A. Rohde, J. H. Christensen, Y. Van de Peer, *et al.* (2003). "Genome-wide characterization of the lignification toolbox in Arabidopsis." Plant Physiology **133**(3): 1051-1071.

Ranjeva, R., G. Alibert and A. M. Boudet (1977). "Metabolism of phenolic compounds in petunia. 6. Studies on biosynthesis of chlorogenic acidand naringenin by isolated chloroplasts." Plant Science Letters **10**(3): 235-242.

Ravelli, R. B. G. and E. F. Garman (2006). "Radiation damage in macromolecular cryocrystallography." Current Opinion in Structural Biology **16**(5): 624-629.

Reger, A. S., J. M. Carney and A. M. Gulick (2007). "Biochemical and crystallographic analysis of substrate binding and conformational changes in Acetyl-CoA synthetase." Biochemistry **46**(22): 6536-6546.

Reger, A. S., R. Wu, D. Dunaway-Mariano and A. M. Gulick (2008). "Structural characterization of a 140 degrees domain movement in the two-step reaction catalyzed by 4-Chlorobenzoate : CoA ligase." Biochemistry **47**(31): 8016-8025.

Reverter, D. and C. D. Lima (2006). "Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates." Nature Structural & Molecular Biology **13**(12): 1060-1068.

Rice-Evans, C., N. Miller and G. Paganga (1997). "Antioxidant properties of phenolic compounds." Trends Plant Sci **2**: 152-159.

Rice-Evans, C. A., N. J. Miller and G. Paganga (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radical Biology and Medicine **20**(7): 933-956.

Ritter, H. and G. E. Schulz (2004). "Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase." Plant Cell **16**(12): 3426-3436.

Ritter, H. and G. E. Schulz (2004). "Structural basis for the entrance into the phenylpropanoid metabolism...".

Rogers, W. J., S. Michaux, M. Bastin and P. Bucheli (1999). "Changes to the content of sugars, sugar alcohols, myo-inositol, carboxylic acids and inorganic anions in developing grains from different varieties of Robusta (Coffea canephora) and Arabica (C. arabica) coffees." Plant Science **149**(2): 115-123.

Rohde, A., K. Morreel, J. Ralph, G. Goeminne, *et al.* (2004). "Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana reveals far-reaching consequences on and carbohydrate metabolism." Plant Cell **16**(10): 2749-2771.

Ruelas, C., M. Tiznado-Hernandez, A. Sanchez-Estrada, M. Robles-Burgueno, *et al.* (2006). "Changes in phenolic acid content during Alternaria alternata in tomato fruit." J Phytopathology **154**(4): 236-244.

Saraste, M., P. R. Sibbald and A. Wittinghofer (1990). "The P-loop - a common motif in ATP-binding and GTP-binding proteins." Trends in Biochemical Sciences **15**(11): 430-434.

Scalbert, A., C. Morand, C. Manach and C. Remesy (2002). "Absorption and metabolism of polyphenols in the gut and impact on health." Biomed Pharmacother **56**(6): 276-282.

Schmelz, S. and J. H. Naismith (2009). "Adenylate-forming enzymes." Current Opinion in Structural Biology **19**(6): 666-671.

Schneider, K., L. Kienow, E. Schmelzer, T. Colby, *et al.* (2005). "A new type of peroxisomal acyl-coenzyme A synthetase from Arabidopsis thaliana has the catalytic capacity to activate biosynthetic precursors of jasmonic acid." Journal of Biological Chemistry **280**(14): 13962-13972.

Schoch, G., S. Goepfert, M. Morant, A. Hehn, *et al.* (2001). "CYP98A3 from Arabidopsis thaliana is a 3 '-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway." Journal of Biological Chemistry **276**(39): 36566-36574.

Schuttelkopf, A. W. and D. M. F. van Aalten (2004). "PRODRG: a tool for high-throughput crystallography of protein-ligand complexes." Acta Crystallographica Section D **60**(8): 1355-1363.

Shahidi, F. and A. Chandrasekara (2010). "Hydroxycinnamates and their in vitro and in vivo antioxidant activities." Phytochemistry Reviews **9**(1): 147-170.

Shomura, Y., I. Torayama, D. Y. Suh, T. Xiang, *et al.* (2005). "Crystal structure of stilbene synthase from Arachis hypogaea." Proteins-Structure Function and Bioinformatics **60**(4): 803-806.

Sondheimer, E. (1964). "Chlorogenic acids and related depsides." Botanical Review **30**(4): 667-712.

Sonnante, G., R. D'Amore, E. Blanco, C. L. Pierri, *et al.* (2010). "Novel Hydroxycinnamoyl-Coenzyme A Quinate Transferase Genes from Artichoke Are Involved in the Synthesis of Chlorogenic Acid." Plant Physiology **153**(3): 1224-1238.

Souza, C. D., B. Barbazuk, S. G. Ralph, J. Bohlmann, *et al.* (2008). "Genome-wide analysis of a land plant-specific acyl : coenzymeA synthetase (ACS) gene family in Arabidopsis, poplar, rice and Physcomitrella." New Phytologist **179**(4): 987-1003.

St-Pierre, B. and V. De Luca (2000). Evolution of acyltransferase genes: Origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. Evolution of Metabolic Pathways. J. T. Romeo, R. Ibrahim, L. Varin and V. DeLuca. Kidlington, Pergamon-Elsevier Science Ltd. **34:** 285-315.

St-Pierre, B., P. Laflamme, A. M. Alarco and V. De Luca (1998). "The terminal O-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer." Plant Journal **14**(6): 703-713.

Stalmach, A., W. Mullen, D. Barron, K. Uchida, *et al.* (2009). "Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption." Drug Metab Dispos **37**(8): 1749-1758.

Stalmach, A., H. Steiling, G. Williamson and A. Crozier (2010). "Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy." Archives of Biochemistry and Biophysics **501**(1): 98-105.

Strack, D., D. PM and H. J. (eds) (1997). "Phenolic metabolism." Plant Biochemistry: 387-416.

Stuible, H. P., D. Buttner, J. Ehlting, K. Hahlbrock, *et al.* (2000). "Mutational analysis of 4-coumarate : CoA ligase identifies functionally important amino acids and verifies its close relationship to other adenylate-forming enzymes." Febs Letters **467**(1): 117-122.

Stuible, H. P. and E. Kombrink (2001). "Identification of the substrate specificity-conferring amino acid residues of 4-coumarate : coenzyme A ligase allows the rational design of mutant enzymes with new catalytic properties." Journal of Biological Chemistry **276**(29): 26893-26897.

Sullivan, M. L. (2009). "A Novel Red Clover Hydroxycinnamoyl Transferase Has Enzymatic Activities Consistent with a Role in Phaselic Acid Biosynthesis." Plant Physiology **150**(4): 1866-1879.

Sullivan, M. L. and R. Zarnowski (2010). "Red clover coumarate 3'-hydroxylase (CYP98A44) is capable of hydroxylating p-coumaroyl-shikimate but not p-coumaroyl-malate: implications for the biosynthesis of phaselic acid." Planta **231**(2): 319-328.

Terwilliger, T. C. (1999). "Reciprocal-space solvent flattening." Acta Crystallographica Section D-Biological Crystallography **55**: 1863-1871.

Terwilliger, T. C. (2000). "Maximum-likelihood density modification." Acta Crystallographica Section D-Biological Crystallography **56**: 965-972.

Terwilliger, T. C. (2003). SOLVE and RESOLVE: Automated structure solution and density modification. Macromolecular Crystallography, Pt D. **374:** 22-37.

Tholl, D. (2006). "Terpene synthases and the regulation, diversity and biological roles of terpene metabolism." Current Opinion in Plant Biology **9**(3): 297-304.

Trott, O. and A. J. Olson (2010). "AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading." Journal of Computational Chemistry **31**(2): 455-461.

Ulbrich, B. and M. H. Zenk (1979). "Partial purification and properties of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase from higher plants." Phytochemistry **18**(6): 929-933.

Unno, H., F. Ichimaida, H. Suzuki, S. Takahashi, *et al.* (2007). "Structural and mutational studies of anthocyanin malonyltransferases establish the features of BAHD enzyme catalysis." J. Biol. Chem. **282**(21): 15812-15822.

Ververidis, F., E. Trantas, C. Douglas, G. Vollmer, *et al.* (2007). "Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes." Biotechnol J **2**(10): 1235-1249.

Villegas, R. J. A. and M. Kojima (1986). "Purification and characterization of hydroxycinnamoyl D-glucose quinate hydroxycinnamoyl transferase in the root of sweet potato, Ipomoea batatas Lam." Journal of Biological Chemistry **261**(19): 8729-8733.

Villegas, R. J. A., T. Shimokawa, H. Okuyama and M. Kojima (1987). "Purification and characterization of chlorogenic acid - chlorogenate caffeoyl transferase in sweet potato roots." Phytochemistry **26**(6): 1577-1581.

Voo, K. S., R. W. Whetten, D. M. Omalley and R. R. Sederoff (1995). "4-Coumarate-coenzyme A ligase from loblolly-pine xylem - Isolation, characterization and complementary DNA cloning." Plant Physiology **108**(1): 85-97.

Walter, T. S., C. Meier, R. Assenberg, K. F. Au, *et al.* (2006). "Lysine methylation as a routine rescue strategy for protein crystallization." Structure **14**(11): 1617-1622.

Wang, Y. and C. T. Ho (2009). "Polyphenolic Chemistry of Tea and Coffee: A Century of Progress." Journal of Agricultural and Food Chemistry **57**(18): 8109-8114.

Waters, E. R. (2003). "Molecular adaptation and the origin of land plants." Mol Phylogenet Evol **29**(3): 456-463.

Wink, M. (2003). "Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective." Phytochemistry **64**(1): 3-19.

Winkel-Shirley, B. (1999). "Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways." Physiologia Plantarum **107**(1): 142-149.

Winkel-Shirley, B. (2001). "Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology, and biotechnology." Plant Physiology **126**(2): 485-493.

Wu, R., A. S. Reger, X. F. Lu, A. M. Gulick, *et al.* (2009). "The Mechanism of Domain Alternation in the Acyl-Adenylate Forming Ligase Superfamily Member 4-Chlorobenzoate: Coenzyme A Ligase." Biochemistry **48**(19): 4115-4125.

Yang, Q., K. Reinhard, E. Schiltz and U. Matern (1997). "Characterization and heterologous expression of hydroxycinnamoyl/benzoyl-CoA:anthranilate N-hydroxycinnamoyl/benzoyltransferase from elicited cell cultures of carnation, Dianthus caryophyllus L." Plant Mol. Biol. **35**(6): 777-789.

Ziouti, A., C. ElModafar, A. ElMandili, E. ElBoustani, *et al.* (1996). "Identification of the caffeoylshikimic acids in the roots of the date palm, principle fungitoxic compounds vis-a-vis Fusarium-oxysporum f sp albedinis." Journal of Phytopathology-Phytopathologische Zeitschrift **144**(4): 197-202.

Sequences:

SEQ ID NO:1
DNA
GTPc103a_Cchct Primer 5'
cagcactagggatccatgaaaatcgaggtgaaggaatcg

SEQ ID NO:2
DNA
GTPc103a_Cchct Primer 3'
tcgtcgatcactcgagtcaaatgtcatacaagaaactctggaa

SEQ ID NO:3
DNA
pGTPc103a_Cchqt Primer 5'
cagcactagggatccatgaagataaccgtgaaggaaaca

SEQ ID NO:4
DNA
pGTPc103a_Cchqt Primer 3'
tcgtcgatcatctagatcagaaatcgtacaggaacctttg

SEQ ID NO:5
DNA
c103g/a104c (H35A)
Forward Primer: atctggtggtgccgaactttgctaccccgagcg

SEQ ID NO:6
DNA
Reverse Primer: cgctcggggtagcaaagttcggcaccaccagat

SEQ ID NO:7
DNA
c457g/a458c (H153A)
Forward Primer: ggtgtgggcatgcgtgctcatgcggcgga

SEQ ID NO:8
DNA
Reverse Primer: tccgccgcatgagcacgcatgcccacacc

SEQ ID NO:9
DNA
c460a/t462t (H154A)
Forward Primer: ggtgtgggcatgcgtcataatgcggcgga

SEQ ID NO:10
DNA
Reverse Primer: tccgccgcattatgacgcatgcccacacc

SEQ ID NO:11
DNA
a470c (D157A)
Forward Primer: catcatgcggcggctggctttagcggc

SEQ ID NO:12
DNA
Reverse Primer: gccgctaaagccagccgccgcatgatg

SEQ ID NO:13
DNA
t754g/a755c (Y252A)
Forward Primer: aagatggcaacaccattagcgctagcagctatgaaatgctgg

SEQ ID NO:14
DNA
Reverse Primer: ccagcatttcatagctgctagcgctaatggtgttgccatctt

SEQ ID NO:15
DNA
t763a (Y255A)
Forward Primer: ccattagctatagcagcaatgaaatgctggccggc

SEQ ID NO:16
DNA

Reverse Primer: gccggccagcatttcattgctgctatagctaatgg

SEQ ID NO:17
DNA
c1120g/g1121a/t1122a (R374E)
Forward Primer: gcattaccagctgggtggaactgccgatccatgatgc

SEQ ID NO:18
DNA
Reverse Primer: gcatcatggatcggcagttccacccagctggtaatgc

SEQ ID NO:19
DNA
c460a/g463c/c464t/g466a/c467g/g468c (H154N/A155L/A156S)
Forward Primer: gggtgtgggcatgcgtcataatctgagcgatggctttagcggcctgcat

SEQ ID NO:20
DNA
Reverse Primer: atgcaggccgctaaagccatcgctcagattatgacgcatgcccacaccc

SEQ ID NO: 21
DNA
>Cchct_pML1
atgaaaatcgaggtgaaggaatcgactatggtgagacctgcccaagaaactcctgggaggaacttgtggaa
ctcgaacgtggacttggtggtgccaaatttccacacccctagcgtctacttctataggccgacgggatcat
cgaatttctttgatgccaaagtgctaaaggacgctttgagccgagcccttgtcccgttctacccaatggct
ggtaggttaaagagagacgaagatgggcggattgagattgagtgcaatggtgaaggcgtgcttttcgtgga
ggccgagtctgatggagtagttgatgatttcggtgactttgcaccaactttagagcttcgtagactcattc
ctgcagttgattattctcaaggaatatcaagctacgctctcctagtgctgcaggtgacatatttcaagtgt
ggtggagtctctcttggtgttggcatgcgacatcatgcagctgatggattttcaggtcttcatttcatcaa
ttcatggtctgatatggcccgtggccttgatgtgaccctgccaccattcatagaccgcaccctcctccgtg
cccgcgatccgccccagcctcaattccagcacattgagtaccaacctcctccagccttaaaagtttccccg
caaactgcaaaatctgactcagttcctgaaactgcggtgtccatcttcaagttaaccagggagcaaatcag
tgccctgaaagccaagtccaaggaagatggaaacaccattagctatagctcctacgaaatgttggcaggcc
atgtatggcgctgtgcttgcaaggcacgaggactcgaagttgatcaaggaacaaaattgtacattgctact
gatggacgggcaagacttaggccttcgctcccacctggctattttggcaatgtcatcttcacggcaacccc
gatagctatagctggtgaccttgaattcaagccagtctggtatgctgccagtaaaatccacgatgcattgg
cgaggatggacaacgactacttaaggtcagctcttgattacttggaattacagcctgatttgaaggccctg
gttcgtggtgcccatactttcaagtgtccaaatctggggatcacaagctgggtaaggttacccatccatga
tgctgattttggctggggtcggcccatatttatgggtcctggtggcatcgcttatgaaggtttaagcttta
tattgcctagtccaaccaatgatggaagcatgtcagtagctatttcattgcagggcgagcacatgaaactc
ttccagagtttcttgtatgacatttga

SEQ ID NO:22
DNA
>CcHCT (Geneart synthetic gene with codons optimised for expression in
E. coli)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag

cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcatacctttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:23
PRT
CcHCT
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:24
DNA
>gene encoding K-mutant_HCT
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcatttttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactggcggttagcccg
cagaccgcggcgagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcatacctttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:25
>K-mutant_HCT (K210A/K217A)
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALAVSP
QTAASDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:26
DNA
>gene encoding HCT_H35A (c103g/a104c)

atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttgctaccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:27
PRT
>HCT_H35A
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFATPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:28
DNA
>gene encoding HCT_H153A (c457g/a458c)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtgctcatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:29
PRT
>HCT_H153A

MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRAHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:30
DNA
>gene encoding HCT_H154N (c460a)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcataatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:31
PRT
>HCT_H154N
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHNAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:32
DNA
>gene encoding HCT_D157A (a470c)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggctggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc

gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:33
PRT
>HCT_D157A
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAAAGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:34
DNA
>gene encoding HCT_Y252A (t754g/a755c)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcatttttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagcgctagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:35
PRT
>HCT_Y252A
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISASSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:36
DNA
>gene encoding HCT_Y255N (t763a)

atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagcaatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:37
PRT
>HCT_Y255N
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSNEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:38
DNA
>gene encoding HCT_R374E (c1120g/g1121a/t1122a)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcatcatgcggcggatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtggaactgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:39
PRT
>HCT_R374E

MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHHAADGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVELPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:40
DNA
>gene encoding HCT_H154N/A155L/A156S
(c460a/g463c/c464t/g466a/c467g/g468c)
atgaaaatcgaagtgaaagaaagcaccatggttcgtccggcgcaggaaaccccgggtcgtaacctgtggaa
cagcaacgtggatctggtggtgccgaactttcataccccgagcgtgtatttttatcgtccgaccggcagca
gcaacttttttgatgcgaaagtgctgaaagatgccctgagccgtgcgctggtgccgttttatccgatggcg
ggtcgtctgaaacgtgatgaagatggccgcattgaaattgaatgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatggcgtggtggatgattttggcgattttgcgccgaccctggaactgcgtcgtctgattc
cggcggtggattatagccagggcattagcagctatgcgctgctggtgctgcaggtgacctattttaaatgc
ggcggtgtgagcctgggtgtgggcatgcgtcataatctgagcgatggctttagcggcctgcattttattaa
cagctggtctgatatggcgcgtggcctggatgttaccctgccgccgtttattgatcgtaccctgctgcgtg
cgcgtgatccgccgcagccgcagtttcagcatattgaatatcaaccgccgccggcactgaaagttagcccg
cagaccgcgaaaagcgatagcgtgccggaaaccgcggtgagcatttttaaactgacccgcgaacaaattag
cgcgctgaaagcgaaaagcaaagaagatggcaacaccattagctatagcagctatgaaatgctggccggcc
atgtgtggcgttgcgcgtgcaaagcgcgtggtctggaagtggatcagggcaccaaactgtatattgcgacc
gatggccgtgcgcgtctgcgtccgagcctgccgccgggttattttggcaacgtgatttttaccgcgacccc
gattgcgattgcgggcgatctggaatttaaaccggtgtggtatgcggcgagcaaaattcatgatgcgctgg
cccgtatggataacgattatctgcgtagcgcgctggattatctggaactgcagccggatctgaaagcgctg
gtgcgtggcgcgcataccttttaaatgcccgaacctgggcattaccagctgggtgcgtctgccgatccatga
tgcggattttggctggggccgtccgatttttatgggtccgggcggtattgcgtatgaaggcctgagcttta
ttctgccgagcccgaccaacgatggcagcatgagcgtggcgattagcctgcagggcgaacatatgaaactg
tttcagagcttcctgtatgatatctaataa

SEQ ID NO:41
PRT
>HCT_H154N/A155L/A156S
MKIEVKESTMVRPAQETPGRNLWNSNVDLVVPNFHTPSVYFYRPTGSSNFFDAKVLKDALSRALVPFYPMA
GRLKRDEDGRIEIECNGEGVLFVEAESDGVVDDFGDFAPTLELRRLIPAVDYSQGISSYALLVLQVTYFKC
GGVSLGVGMRHNLSDGFSGLHFINSWSDMARGLDVTLPPFIDRTLLRARDPPQPQFQHIEYQPPPALKVSP
QTAKSDSVPETAVSIFKLTREQISALKAKSKEDGNTISYSSYEMLAGHVWRCACKARGLEVDQGTKLYIAT
DGRARLRPSLPPGYFGNVIFTATPIAIAGDLEFKPVWYAASKIHDALARMDNDYLRSALDYLELQPDLKAL
VRGAHTFKCPNLGITSWVRLPIHDADFGWGRPIFMGPGGIAYEGLSFILPSPTNDGSMSVAISLQGEHMKL
FQSFLYDI--

SEQ ID NO:42
DNA
>Cchqt_pML2
atgaagataaccgtgaaggaaacagcaatggttcgtccagcccaaccaacacccacaaagaggctatggaa
ctcgaatttggatcttctggtagcaagaattcatatcttgaccgtctacttctacaagcctaatggttctg
ctaatttctttgatacccgtgtgctcaaagaagcattgagcaatgttcttgtctctttctaccccatggct
gggagattagcaagagatgaagaaggaaggattgagatcgactgtaacggtgaaggtgtactctttgttga
ggctgaatcagactcgagtgtcgatgattttggtgattttgcaccaagtttggagctcaggaggctcatcc
cgaccgtcgattgttctggtgacatttcttcttacccccctcgttatttttccaggtaactcgtttcaagtgt
ggagcagcagctcttggagctggggtgcagcacaatttatcagatggcgtttcatctctgcacttcatcaa
tacatggtcagacatagctcgtggcctcaccattgctgtccctccgttcattgaccggacacttatccgtg
ctagggaccctcccgtgcctgtattcgaacatgtcgaatattatccgccccctcaactaaaatcagattca
agaattcaagaactcaaaacaggccctagggcaagtaccacagctgtactaaaaatcacacctgaacaact
tagccagcttaaagctaaggccaacaatgagggcagcacttatgagatcttagcagcacatatatggcgta

ctgcatgcaaagcacgtggcctcaccaatgatcaatccaccaagctgtacgttgccactgatggccggtct
aggctgattcctcccctgcctcctggctatctaggtaatgtggtcttcactgcaactccaattgctgaatc
cagtaatcttcaatcagagcccttgaccaattctgcaaaaagaatccacaacgccttggcaagaatggata
atgattacttaagatcagccctggattatcttgaaattcaacctgatttgtctgctttggttagagggcct
cggcattttgctagtcctaatttgaatatcaatagttggacaaggcttccgtttcatgatgcagactttgg
ctggggcagacctattcatatagggccggcaatcattctttatgagggtacggtatatgtattgccaagtc
caggcaaagacaggactttatcgttagctgtgtgcttagatgccgatcacatgccactcttccaaaggttc
ctgtacgatttctga

SEQ ID NO:43
DNA
>Cchqt (Geneart synthetic gene with codons optimised for expression in
E. coli)
atgaaaatcaccgtgaaagaaaccgcgatggtgcgtccggcgcagccgaccccgaccaaacgtctgtggaa
cagcaacctggatctgctggttgcgcgtattcatattctgaccgtgtacttctataaaccgaacggcagcg
cgaactttttgatacccgcgtgctgaaagaagccctgagcaacgttctggtgagcttttatccgatggcg
ggtcgtctggcccgtgatgaagaaggccgtattgaaattgattgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatagcagcgtggatgattttggcgattttgcgccgagcctggaactgcgtcgtctgattc
cgaccgtggattgcagcggcgacattagcagctatccgctggtgatttttcaggtgacccgttttaaatgc
ggcgcagcagcgctgggtgcgggtgtgcagcataacctgagcgatggcgtgagcagcctgcattttattaa
cacctggtctgatattgcgcgtggcctgaccattgcggtgccgccgtttattgatcgtaccctgattcgtg
cgcgtgatccgccggtgccggtgtttgaacatgtggaatattatccgccgccgcagctgaaaagcgatagc
cgtattcaggaactgaaaaccggtccgcgtgcgagcaccaccgcggtgctgaaaattaccccggaacagct
gtctcagctgaaagcgaaagcgaacaacgaaggcagcacctatgaaattctggccgcgcatatttggcgta
ccgcgtgcaaagcgcgcggtctgaccaacgatcagagcaccaaactgtatgtggcgaccgatggccgtagc
cgcctgattccgccgctgccgccgggttatctgggcaacgtggtgtttaccgcgaccccgattgcggaaag
cagcaacctgcagagcgaaccgctgaccaacagcgcgaaacgtattcataacgcgctggcccgtatggata
acgattatctgcgtagcgcgctggattatctggaaattcagccggatctgagcgcgctggttcgtggcccg
cgtcattttgcgagcccgaacctgaacattaacagctggacccgtctgccgtttcatgatgcggattttgg
ctggggccgtccgattcatattggtccggcgattattctgtatgaaggcaccgtgtatgtgctgccgagcc
cgggcaaagatcgcaccctgagcctggccgtgtgcctggatgcggatcatatgccgctgtttcagcgtttt
ctgtatgatttctaa

SEQ ID NO:44
PRT
>CcHQT
MKITVKETAMVRPAQPTPTKRLWNSNLDLLVARIHILTVYFYKPNGSANFFDTRVLKEALSNVLVSFYPMA
GRLARDEEGRIEIDCNGEGVLFVEAESDSSVDDFGDFAPSLELRRLIPTVDCSGDISSYPLVIFQVTRFKC
GAAALGAGVQHNLSDGVSSLHFINTWSDIARGLTIAVPPFIDRTLIRARDPPVPVFEHVEYYPPPQLKSDS
RIQELKTGPRASTTAVLKITPEQLSQLKAKANNEGSTYEILAAHIWRTACKARGLTNDQSTKLYVATDGRS
RLIPPLPPGYLGNVVFTATPIAESSNLQSEPLTNSAKRIHNALARMDNDYLRSALDYLEIQPDLSALVRGP
RHFASPNLNINSWTRLPFHDADFGWGRPIHIGPAIILYEGTVYVLPSPGKDRTLSLAVCLDADHMPLFQRF
LYDF

SEQ ID NO:45
DNA
>gene encoding K-mutant_HQT (Geneart)
atgaaaatcaccgtgaaagaaaccgcgatggtgcgtccggcgcagccgaccccgaccaaacgtctgtggaa
cagcaacctggatctgctggttgcgcgtattcatattctgaccgtgtacttctataaaccgaacggcagcg
cgaactttttgatacccgcgtgctgaaagaagccctgagcaacgttctggtgagcttttatccgatggcg
ggtcgtctggcccgtgatgaagaaggccgtattgaaattgattgcaacggcgaaggcgtgctgtttgtgga
agcggaaagcgatagcagcgtggatgattttggcgattttgcgccgagcctggaactgcgtcgtctgattc
cgaccgtggattgcagcggcgacattagcagctatccgctggtgatttttcaggtgacccgttttaaatgc
ggcgcagcagcgctgggtgcgggtgtgcagcataacctgagcgatggcgtgagcagcctgcattttattaa
cacctggtctgatattgcgcgtggcctgaccattgcggtgccgccgtttattgatcgtaccctgattcgtg
cgcgtgatccgccggtgccggtgtttgaacatgtggaatattatccgccgccgcagctggcgagcgatagc
cgtattcaggaactggcgaccggtccgcgtgcgagcaccaccgcggtgctgaaaattaccccggaacagct

gtctcagctgaaagcgaaagcgaacaacgaaggcagcacctatgaaattctggccgcgcatatttggcgta
ccgcgtgcaaagcgcgcggtctgaccaacgatcagagcaccaaactgtatgtggcgaccgatggccgtagc
cgcctgattccgccgctgccgccgggttatctgggcaacgtggtgtttaccgcgaccccgattgcggaaag
cagcaacctgcagagcgaaccgctgaccaacagcgcgaaacgtattcataacgcgctggcccgtatggata
acgattatctgcgtagcgcgctggattatctggaaattcagccggatctgagcgcgctggttcgtggcccg
cgtcattttgcgagcccgaacctgaacattaacagctggacccgtctgccgtttcatgatgcggattttgg
ctggggccgtccgattcatattggtccggcgattattctgtatgaaggcaccgtgtatgtgctgccgagcc
cgggcaaagatcgcaccctgagcctggccgtgtgcctggatgcggatcatatgccgctgtttcagcgtttt
ctgtatgatttctaa

SEQ ID NO:46
PRT
>K-mutant_HQT (K210A/K219A)
MKITVKETAMVRPAQPTPTKRLWNSNLDLLVARIHILTVYFYKPNGSANFFDTRVLKEALSNVLVSFYPMA
GRLARDEEGRIEIDCNGEGVLFVEAESDSSVDDFGDFAPSLELRRLIPTVDCSGDISSYPLVIFQVTRFKC
GAAALGAGVQHNLSDGVSSLHFINTWSDIARGLTIAVPPFIDRTLIRARDPPVPVFEHVEYYPPPQLASDS
RIQELATGPRASTTAVLKITPEQLSQLKAKANNEGSTYEILAAHIWRTACKARGLTNDQSTKLYVATDGRS
RLIPPLPPGYLGNVVFTATPIAESSNLQSEPLTNSAKRIHNALARMDNDYLRSALDYLEIQPDLSALVRGP
RHFASPNLNINSWTRLPFHDADFGWGRPIHIGPAIILYEGTVYVLPSPGKDRTLSLAVCLDADHMPLFQRF
LYDF-

SEQ ID NO:47
PRT
>Cc4CL2
MILLPNSPEFVFAFLGASFRGAISTMANPYFTSAEVIKQAKASNAKLIITQGWYVEKVMDYACENGVKVVC
IDSAPEGCLRFSELTEADEREMLDVEISPEDVVALPYSSGTTGLPKGVMLTHKGLVTSVAQQVDGENPNFY
IHNQVMMCVLPLFHIYSLNSILLCGLRAGTTILIMQKFDIIPFLELIQKYKVTTGPFVPPIVLAIAKSPEV
DKYDLSSVKTVMSGAAPLGKELEDAVRTKFPKAKLGQGYGMTEAGPVLAMCSAFAKDPFEVKSGGCGSVVR
NAEMKIVDPETGSSLPRNQPGEICIRGDQIMKGYLDDPEATKATIDEDGWLHTGDVGYIDEDDELFIVDRL
KELIKYKGFQVAPAELEALLLAHSDISDAAVVPMKDDAAGEVPVAFVVKSKDSNITEDEIKEYIKKQVIFY
KRINRVFFVDAIPKSPSGKILRKDLRARLAAGVPK-

CLAIMS

What is Claimed:

1. A recombinant *Coffea* wild-type HCT, HQT, or 4CL enzyme expressed in a microbial cell and having enzyme activity substantially identical to a corresponding enzyme isolated from a *Coffea spp.* plant; optionally wherein the plant is *C. canephora*; optionally wherein the microbial cell is *E. coli*, *S. cerevisiae*, or a food-grade lactic acid bacteria.
2. The recombinant enzyme according to claim 1 that is a 4CL enzyme capable of catalysing the production of a hydroxycinnamic acid (HCA)-CoA thioester from an HCA in the presence of coenzyme A (CoA), a divalent metal ion, and adenosine triphosphate (ATP); optionally which catalyses the production of a CoA thioester from at least cinnamic acid, coumaric acid, caffeic acid, ferulic acid, and sinapic acid.; optionally which is capable of converting at least about 80% of cinnamic acid, coumaric acid, caffeic acid, or ferulic acid to the corresponding HCA-CoA; optionally which is capable of producing *in vitro* a HCA-CoA thioester substantially free from the HCA acid precursor; optionally wherein the recombinant enzyme has an amino acid of SEQ ID NO: 23 or 44 or 47.
3. The recombinant enzyme according to claim 1 or claim 2 that is a HCT or HQT enzyme capable of catalysing *in vivo* in the microorganism or *in vitro* the production of a chlorogenic acid species or dactylifric acid species from a HCA-CoA thioester and an acyl acceptor molecule; optionally wherein the HCA-CoA thioester comprises one or more of the thioesters coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA, feruloyl-CoA, or sinapoyl-CoA; optionally wherein the acyl acceptor comprises quinic acid, shikimic acid, or a mixture thereof.
4. A mutant plant HCT enzyme comprising an amino acid sequence that is at least 80% identical to that of a corresponding wild-type HCT enzyme found in the same species of plant, and contains an altered amino acid residue in one or more positions relative to the wild-type HCT enzyme, wherein the mutant can bind at least one substrate or catalyze the formation of at least one product in either the forward or reverse direction; optionally wherein the altered amino acid residue is at a position corresponding to an amino acid in a reference amino acid sequence that is the *Coffea canephora* HCT enzyme sequence (SEQ ID NO:23) and the amino acid is W23, N26, V27, D28, L29, V30, V31, P32, N33, F34, H35, T36, P37, S38, V39, Y40, P110, R115, G143, G144, V149, G150, M151, R152,

H153, H154, A155, A156, D157, G158, F159, S160, G161, L162, H163, F164, I165, Y203, K210, K217, L231, N248, Y252, Y255, L272, V274, D275, Q276, L280, Y281, I282, A283, T284, D285, R289, L294, N301, V302, I303, F304, T305, L331, L346, K353, L355, V356, R357, G358, A359, H360, T361, F362, K363, C364, P365, N366, L367, G368, I369, T370, S371, W372, V373, R374, L375, P376, I377, G383, M390, G391, P392, G393, G394, I395, A396, Y397, E398, G399, L400, S401, or F402, or any combinations thereof; optionally comprising an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HCT sequence (SEQ ID NO:23); optionally comprising one or more of the mutations H35A, H153A, H154A, A155L, A156S, D157A, Y252A, Y255A, R374E, K210A/K217A, or H154N/A155L/A156S; optionally comprising an amino acid sequence that is any of SEQ ID NOs:25, 27, 29, 31, 33, 35, 37, 39, or 41.

5. The mutant HCT according to claim 4 which, relative to the wild-type HCT, has improved ability to bind at least one substrate, or to catalyze the formation of at least one product in either the forward or backward direction, or greater resistance to proteolysis; optionally which shows a substrate preference for shikimic acid species relative to the wild-type enzyme; optionally which shows an improved ability to catalyze the formation of diCQA relative to the wild-type enzyme.

6. A mutant plant HQT enzyme comprising an amino acid sequence that is at least 80% identical to that of a corresponding wild-type HQT enzyme found in a plant, but which contains an altered amino acid residue in one or more positions relative to the wild-type HQT enzyme, wherein the mutant can bind at least one substrate or catalyze the formation of at least one product in either the forward or reverse direction; optionally wherein the altered amino acid residue is at a position corresponding to an amino acid in a reference amino acid sequence that is the *Coffea canephora* HQT enzyme sequence (SEQ ID NO:44) and the amino acid is W23, N26, I27, D28, L29, L30, V31, A32, R33, I34, H35, I36, L37, T38, V39, Y40, P110, R115, G143, A144, A149, G150, V151, Q152, H153, N154, L155, S156, D157, G158, V159, S160, S161, L162, H163, F164, I165, Y203, K210, L217, I231, N245, E246, G247, Y252, L272, N274, D275, Q276, L280, Y281, V282, A283, T284, D285, R289, L294, N301, V302, I303, F304, T305, L331, L346, S353, L355, V356, R357, G358, D359, R360, H361, F362, A363, S364, P365, N366, L367, N368, I369, N370, S371,

W372, T373, R374, L375, P376, F377, G383, I390, G391, P392, A393, I394, I395, L396, Y397, E398, G399, T400, V401, or Y402; optionally comprising an alteration of the amino acid corresponding to H35, H153, H154, A155, A156, D157, K210, K217, Y252, Y255, or R374 of the reference HQT sequence (SEQ ID NO:44); optionally comprising one or more of the mutations H35A, H153A, N154A, L155A, S156A, D157A, Y252A, R374E, K210A, or combinations thereof; optionally comprising an amino acid sequence that is SEQ ID NO: 46.

7. The mutant HQT according to claim 6, which relative to the wild-type HQT has improved ability to bind at least one substrate, or to catalyze the formation of at least one product in either the forward or backward direction, or greater resistance to proteolysis; optionally which shows a substrate preference for quinic acid species relative to the wild-type enzyme; optionally which shows an improved ability to catalyze the formation of diCQA relative to the wild-type enzyme.
8. A composition comprising one or more recombinant enzymes according to any of claims 1-3, one or more mutant HCTs according to claim 4 or 5, or one or more mutant HQTs according to claim 6 or 7, adapted for *in vitro* synthesis of one or more chlorogenic acid species or dactylifric acid species; optionally comprising substrates and cofactors sufficient for the production of one or more chlorogenic acid species or dactylifric acid species; optionally wherein one or more of the enzymes is immobilised on or covalently bound to a matrix; optionally adapted for *in vitro* production of a HCA-CoA thioester catalysed by a 4CL enzyme, prior to *in vitro* production of the chlorogenic acid species or dactylifric acid species.
9. A method of producing a chlorogenic acid or dactylifric acid species *in vitro* comprising the steps of

   a) providing an HCT or HQT or both;

   b) providing substrates and cofactors sufficient to form a chlorogenic acid or dactylifric acid species in the presence of an HCT or HQT or both; and

   c) contacting the substrates and cofactors with the HCT or HQT, or both, under conditions permitting enzymatic activity, for a time sufficient to permit the formation of product that is a chlorogenic acid or dactylifric acid species;

wherein the method optionally comprises an additional step wherein a HCA-CoA thioester is produced using a 4CL enzyme.

10. A method of producing a chlorogenic acid or dactylifric acid species *in vitro* comprising the steps of

a) providing an HCT, and optionally, an HQT or 4CL or both;

b) providing one or more HCA thioesters, an acyl acceptor, CoA, and a first chlorogenic acid or dactylifric acid species;

c) contacting the one or more HCA thioesters, acyl acceptor, CoA, and first chlorogenic acid or dactylifric acid species with the HCT under conditions permitting enzymatic activity, and for a time sufficient to permit the formation of product that is a second chlorogenic acid or dactylifric acid species;

optionally wherein the first chlorogenic acid or dactylifric acid species is catalytically converted into a second chlorogenic acid or dactylifric acid species.

11. A food product comprising one or more exogenous chlorogenic acid or dactylifric acid species; optionally wherein the chlorogenic acid or dactylifric acid species are produced *in vitro*; optionally wherein the food product is a coffee product; optionally wherein the coffee product is a soluble coffee product wherein the exogenous chlorogenic acid or dactylifric acid species provide a functional ingredient that improves quality or provides a health benefit.

12. The food product according to claim 11, wherein the exogenous chlorogenic acid or dactylifric acid species directly or indirectly provide a desirable flavour attribute, aroma attribute, or colour attribute; optionally wherein the exogenous chlorogenic acid or dactylifric acid species contribute antioxidant properties.

13. The food product according to claim 11 or 12, wherein the exogenous chlorogenic acid or dactylifric acid species comprise mono- and diester CGA or CSA species; optionally wherein the exogenous chlorogenic acid comprises 3,4, caffeoylquinic acid at levels that improve the flavour and aroma of the food product as determined by sensory evaluation; optionally wherein the exogenous chlorogenic acid comprises 3,4, caffeoylquinic acid but is substantially free of 5-feruloylquinic acid.

14. A recombinant microorganism comprising a gene for an expressible exogenous enzyme that is a *Coffea* HCT or HQT; wherein the microorganism expresses the enzyme; optionally

wherein the recombinant microorganism is a food-grade lactic acid bacteria; optionally wherein the recombinant microorganism can produce a chlorogenic acid or dactylifric acid species in an amount not naturally produced in a comparable microorganism that does not express the enzyme.

15. A transgenic plant comprising a heterologous gene for an expressible exogenous enzyme that is a *Coffea* HCT or HQT; wherein the plant expresses the enzyme; optionally wherein the ttransgenic plant produces less lignin than an equivalent plant that does not comprise the heterologous gene; optionally wherein the transgenic plant comprises substantially equivalent or superior biomass and growth characteristics as an equivalent plant that does not comprise the heterologous gene, under equivalent growth conditions.

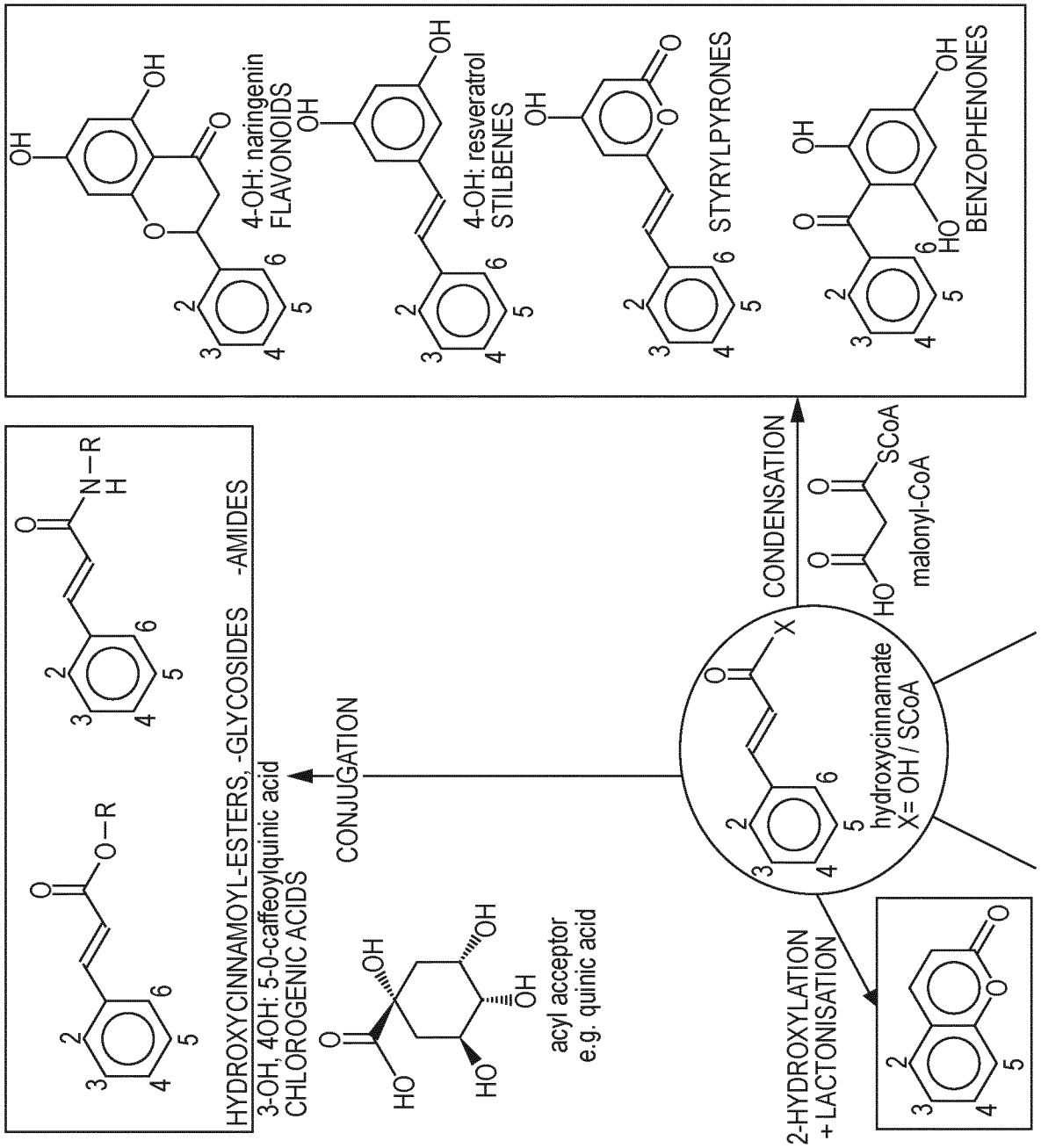

HYDROXYCINNAMOYL-ESTERS, -GLYCOSIDES -AMIDES
3-OH, 4OH: 5-0-caffeoylquinic acid
CHLOROGENIC ACIDS
CONJUGATION
acyl acceptor
e.g. quinic acid
hydroxycinnamate
X= OH / SCoA
CONDENSATION
malonyl-CoA
2-HYDROXYLATION
+ LACTONISATION
4-OH: naringenin
FLAVONOIDS
4-OH: resveratrol
STILBENES
STYRYLPYRONES
BENZOPHENONES

FIG. 1

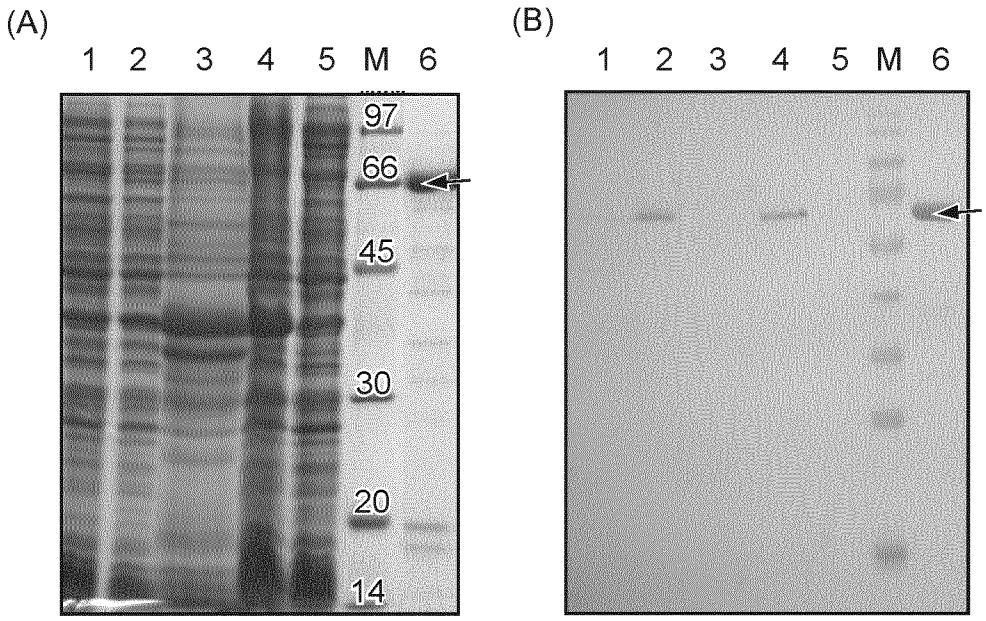

(A)
1 2 3 4 5 M 6
97
66
45
30
20
14
(B)
1 2 3 4 5 M 6

FIG. 2

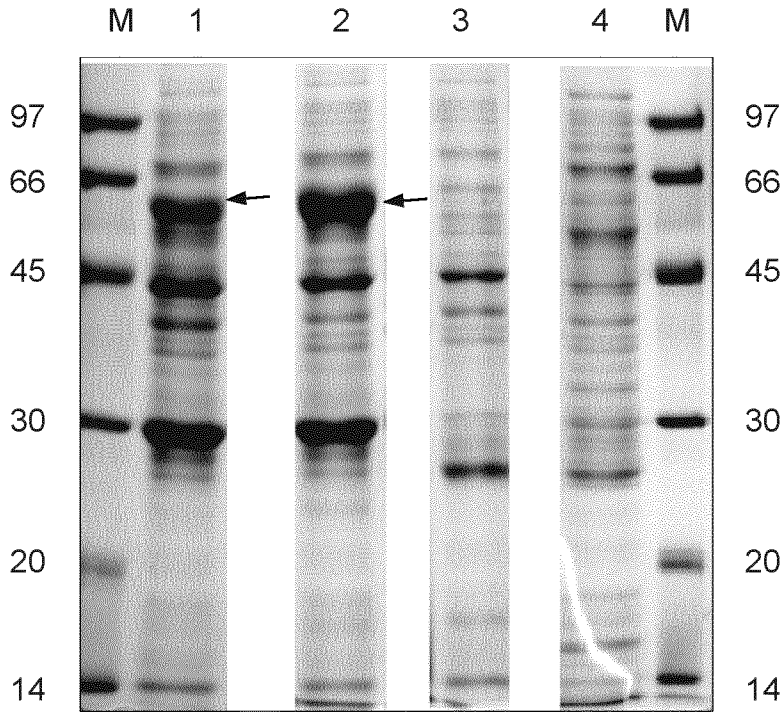

M 1 2 3 4 M
97
66
45
30
20
14
97
66
45
30
20
14

FIG. 3

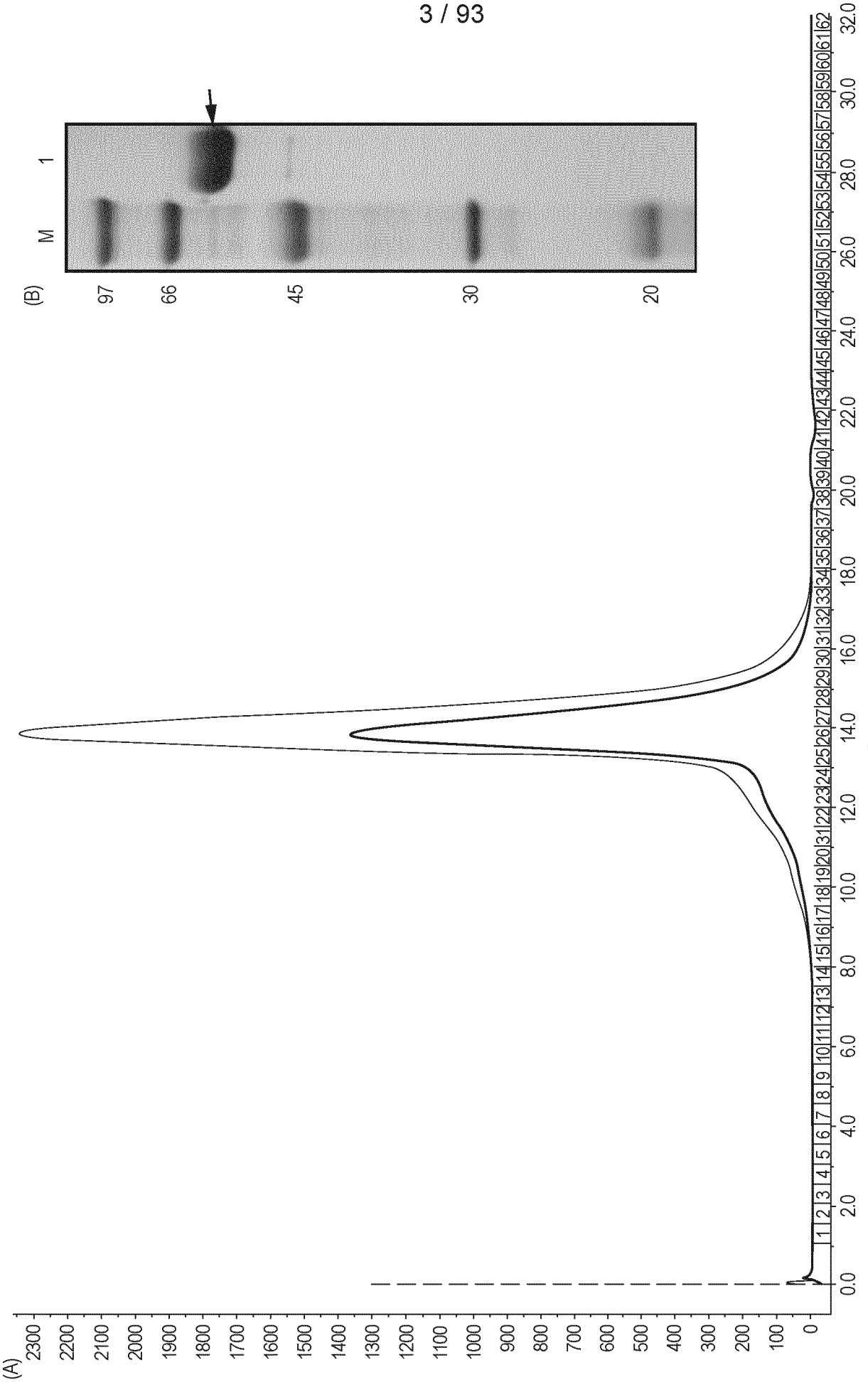


FIG. 4

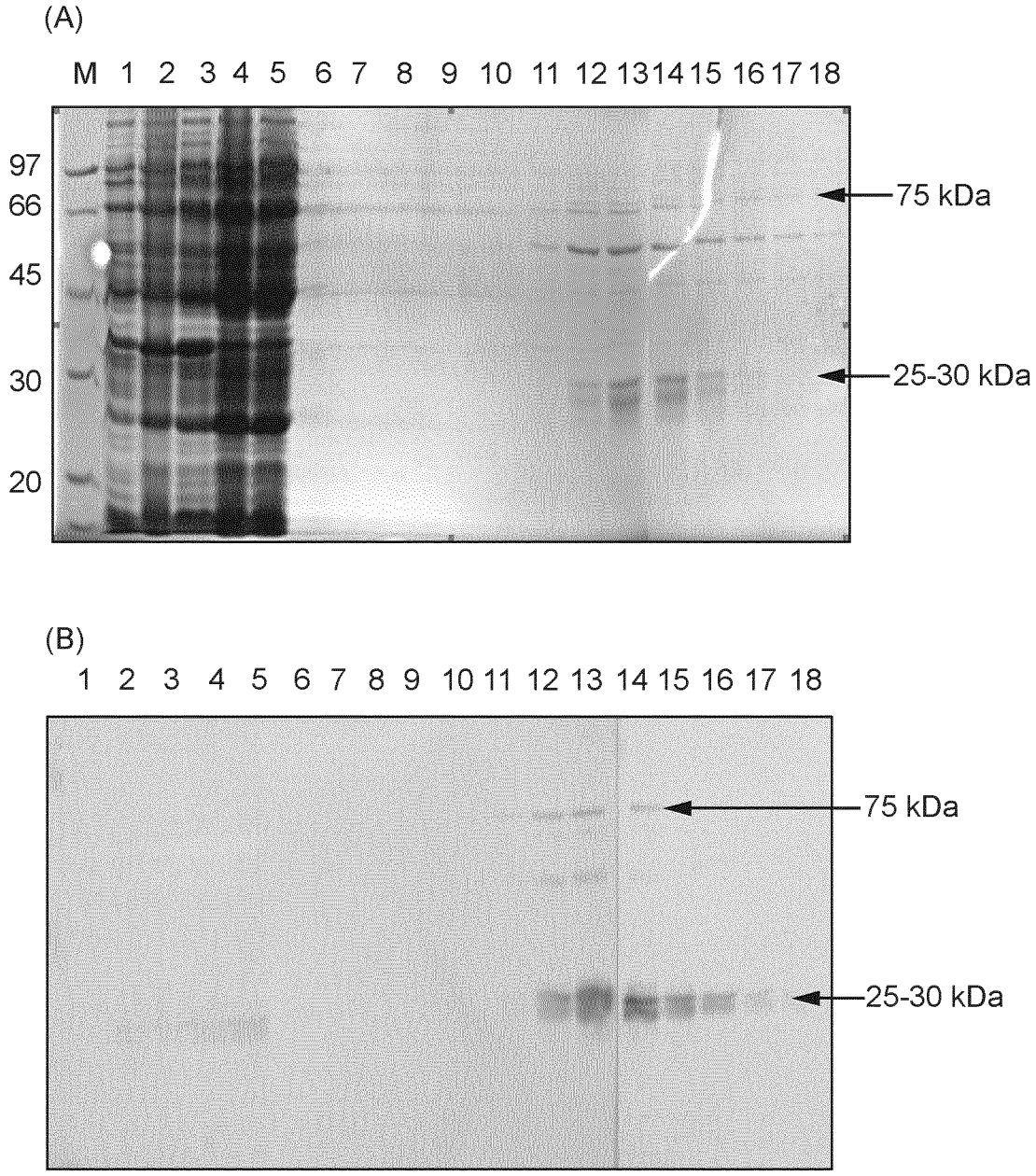

(A)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
97
66
45
30
20
75 kDa
25-30 kDa
(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
75 kDa
25-30 kDa

FIG. 5

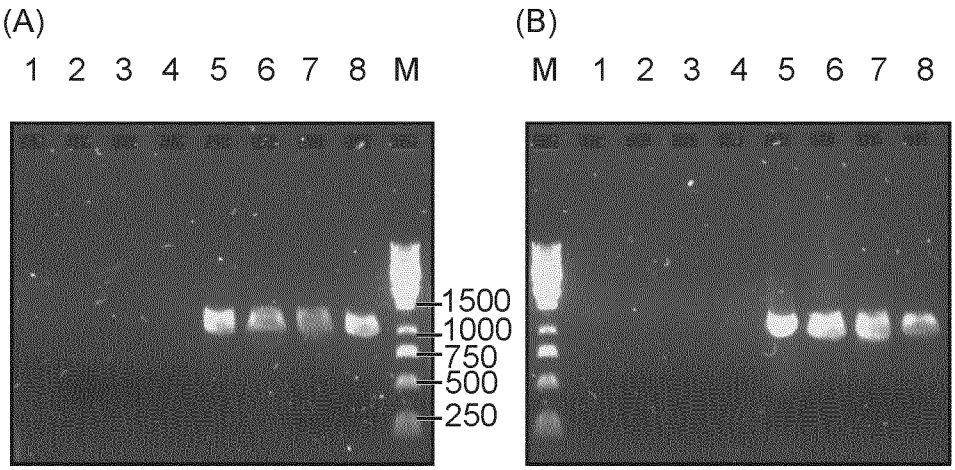

(A)
1 2 3 4 5 6 7 8 M
(B)
M 1 2 3 4 5 6 7 8
1500
1000
750
500
250

FIG. 6

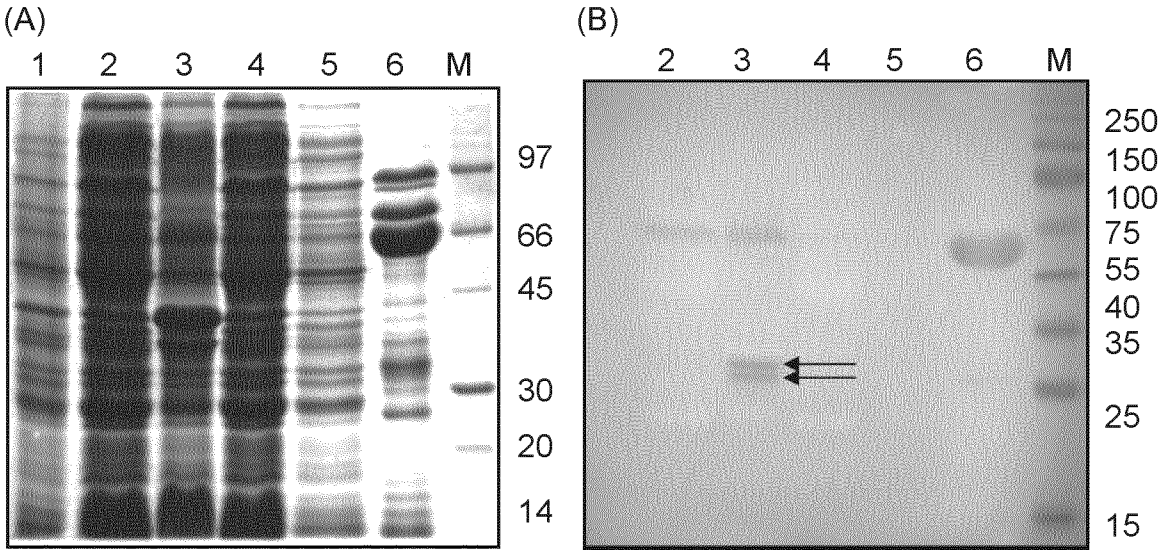

(A)
1 2 3 4 5 6 M
97
66
45
30
20
14
(B)
2 3 4 5 6 M
250
150
100
75
55
40
35
25
15

FIG. 7

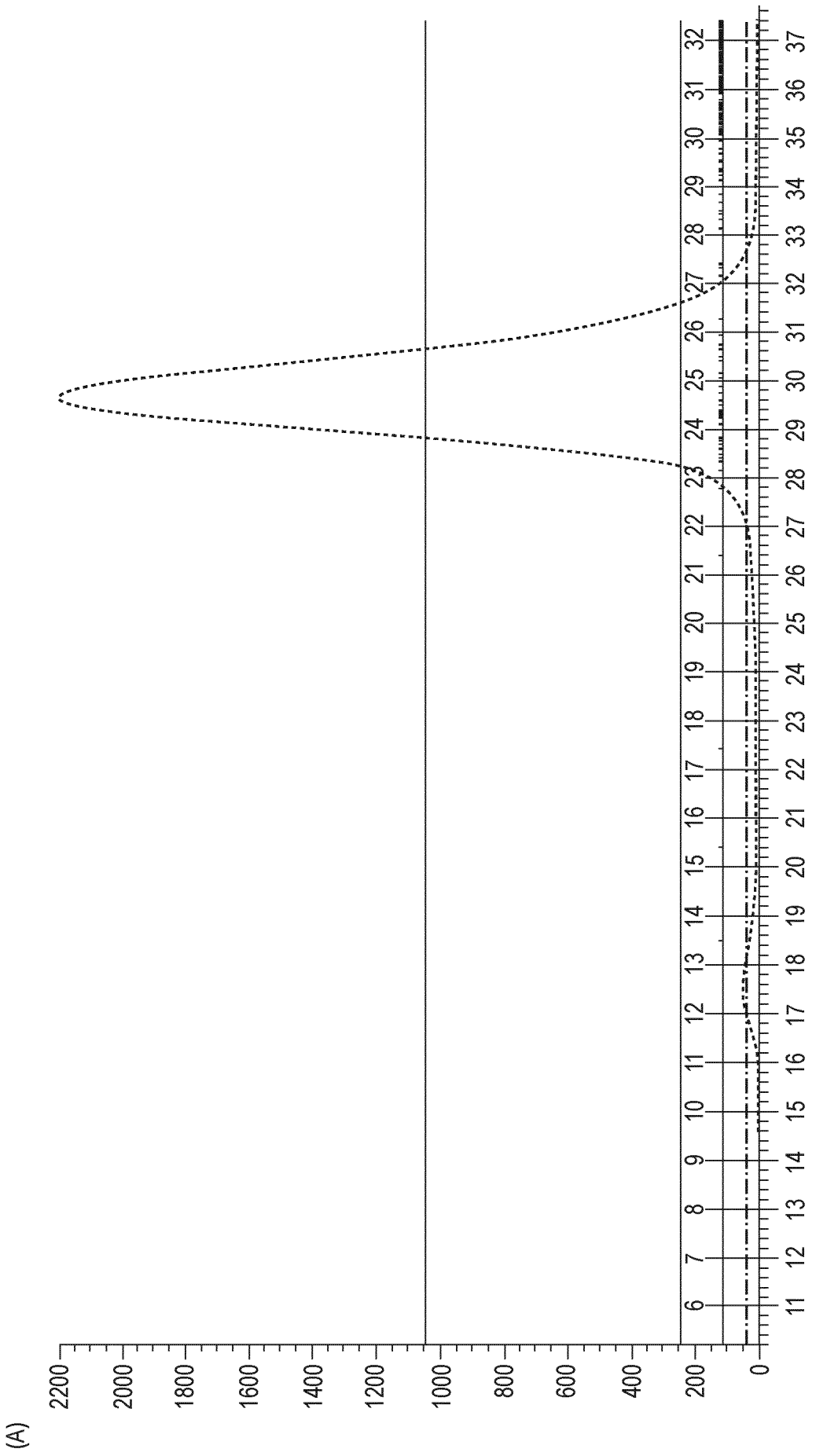

(A)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

FIG. 8

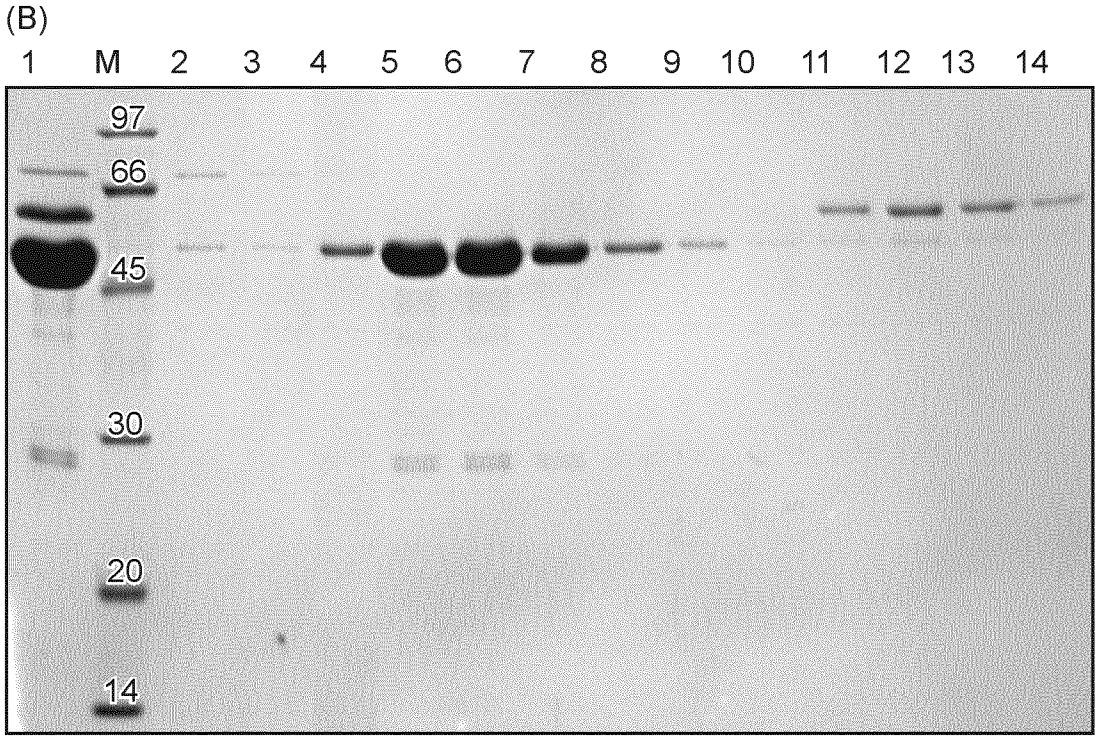


FIG. 8 Cont'd

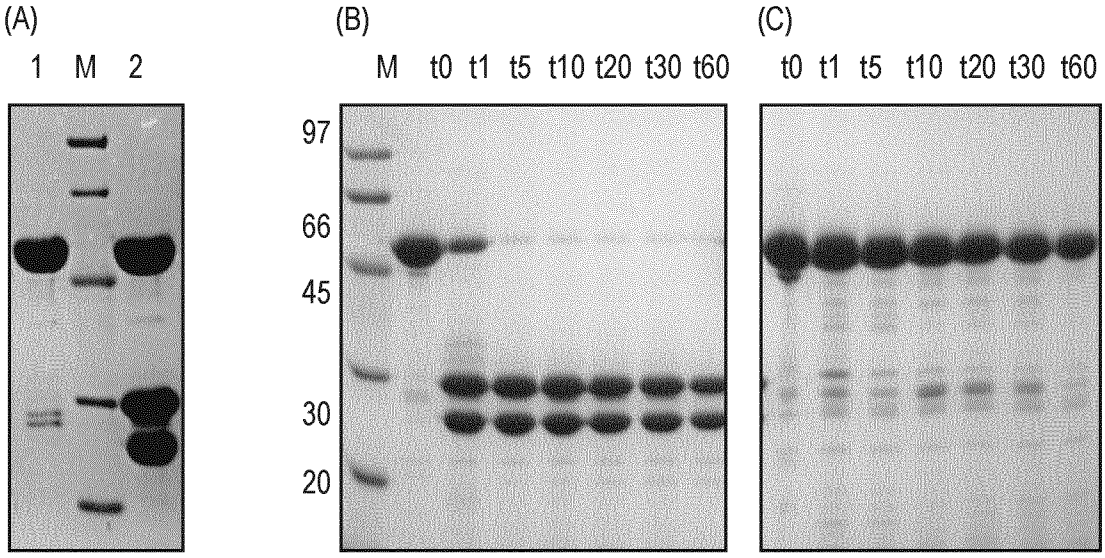

(A)
1 M 2
(B)
M t0 t1 t5 t10 t20 t30 t60
97
66
45
30
20
(C)
t0 t1 t5 t10 t20 t30 t60

FIG. 9

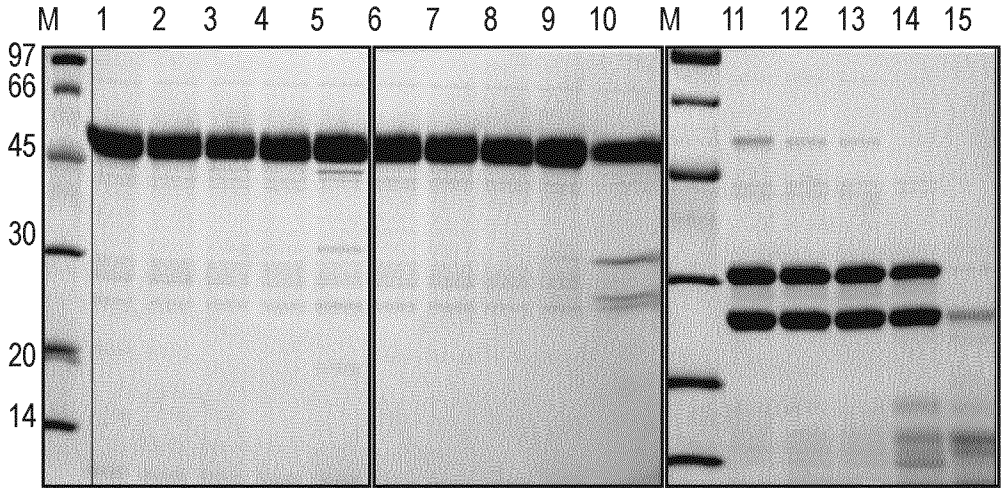

M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15
97
66
45
30
20
14

FIG. 10

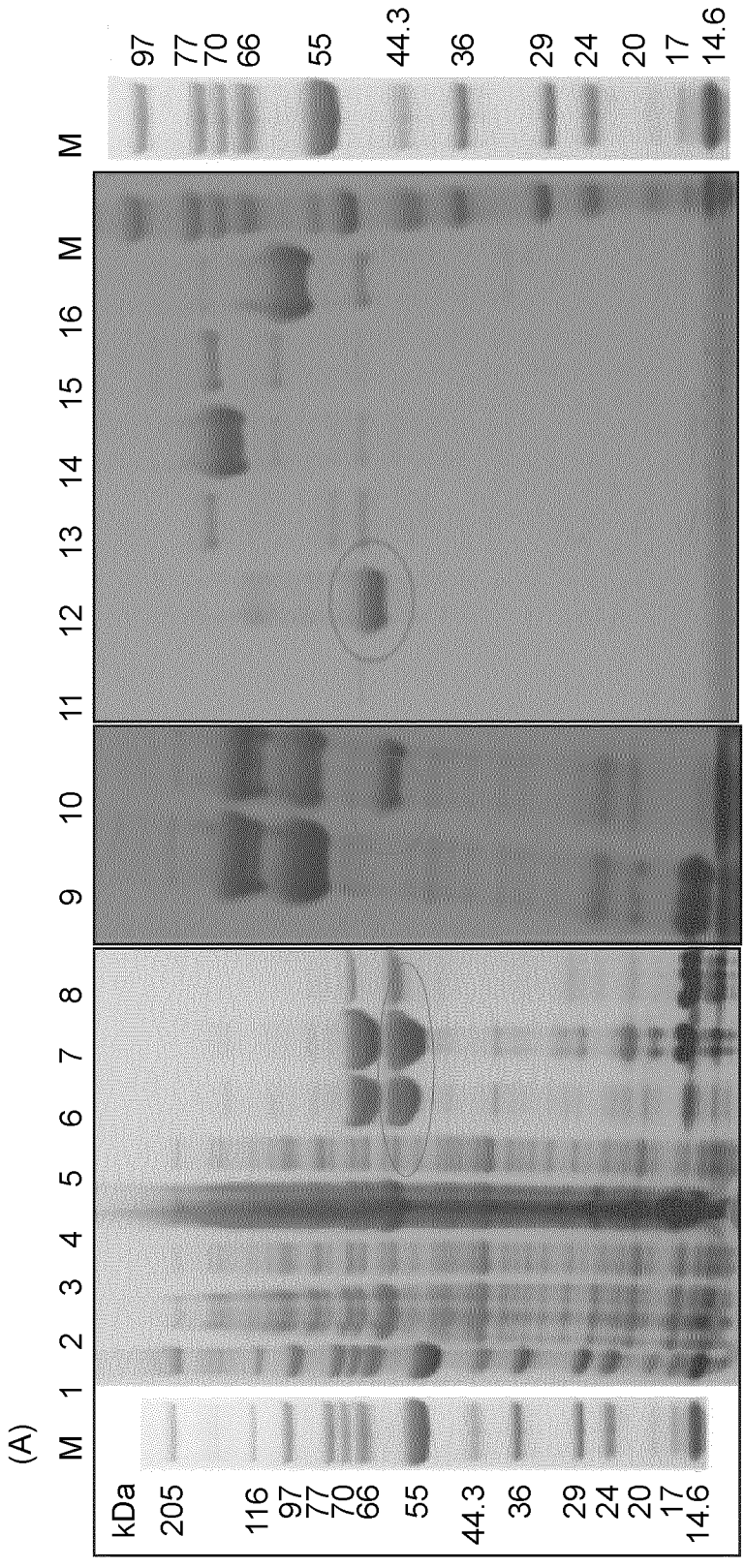

(A)
kDa
205
116
97
77
70
66
55
44.3
36
29
24
20
17
14.6
M
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
M
M
97
77
70
66
55
44.3
36
29
24
20
17
14.6

FIG. 11

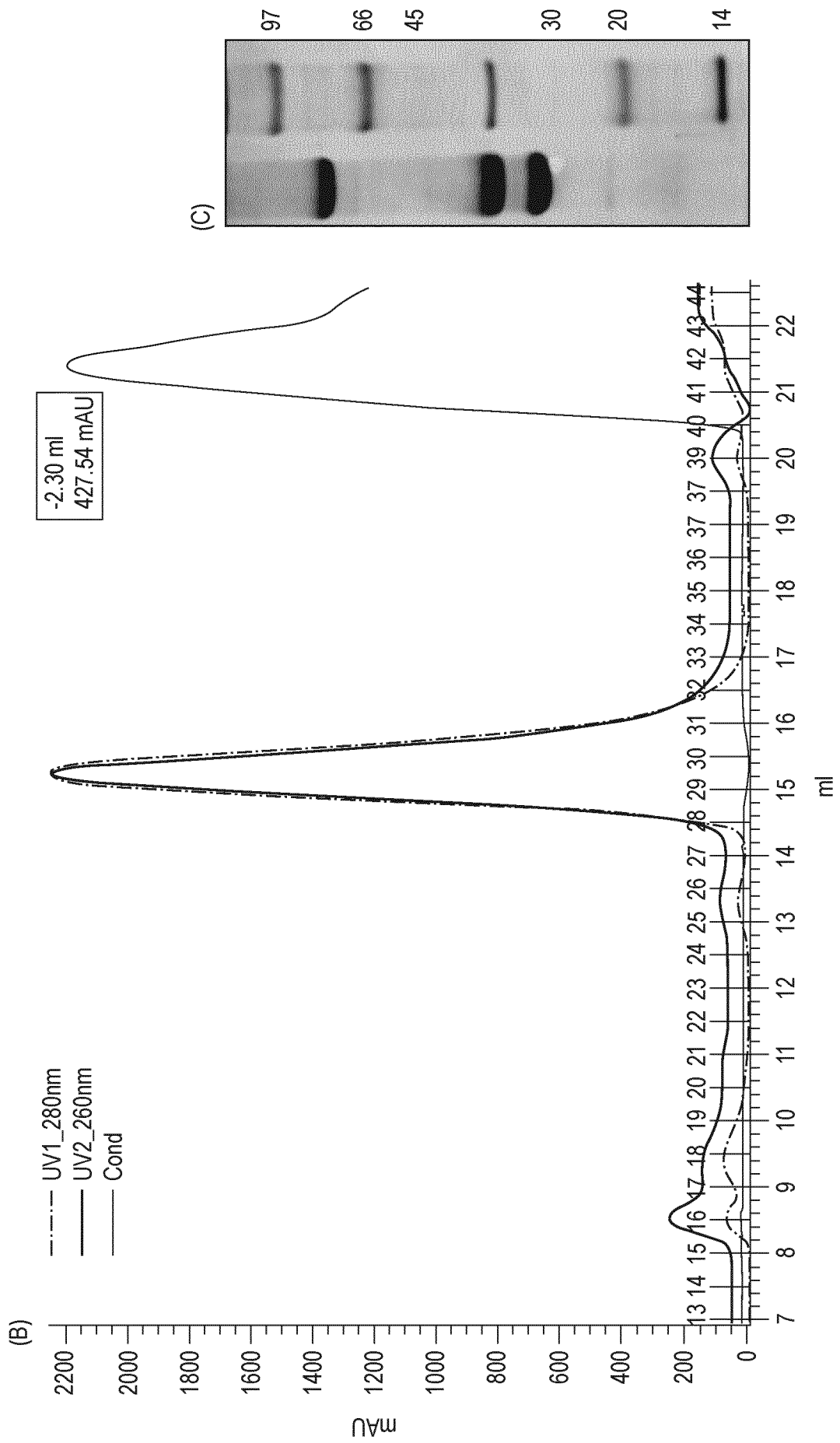

(C)
97
66
45
30
20
14
(B)
-2.30 ml
427.54 mAU
UV1_280nm
UV2_260nm
Cond
mAU
ml

FIG. 11 Cont'd

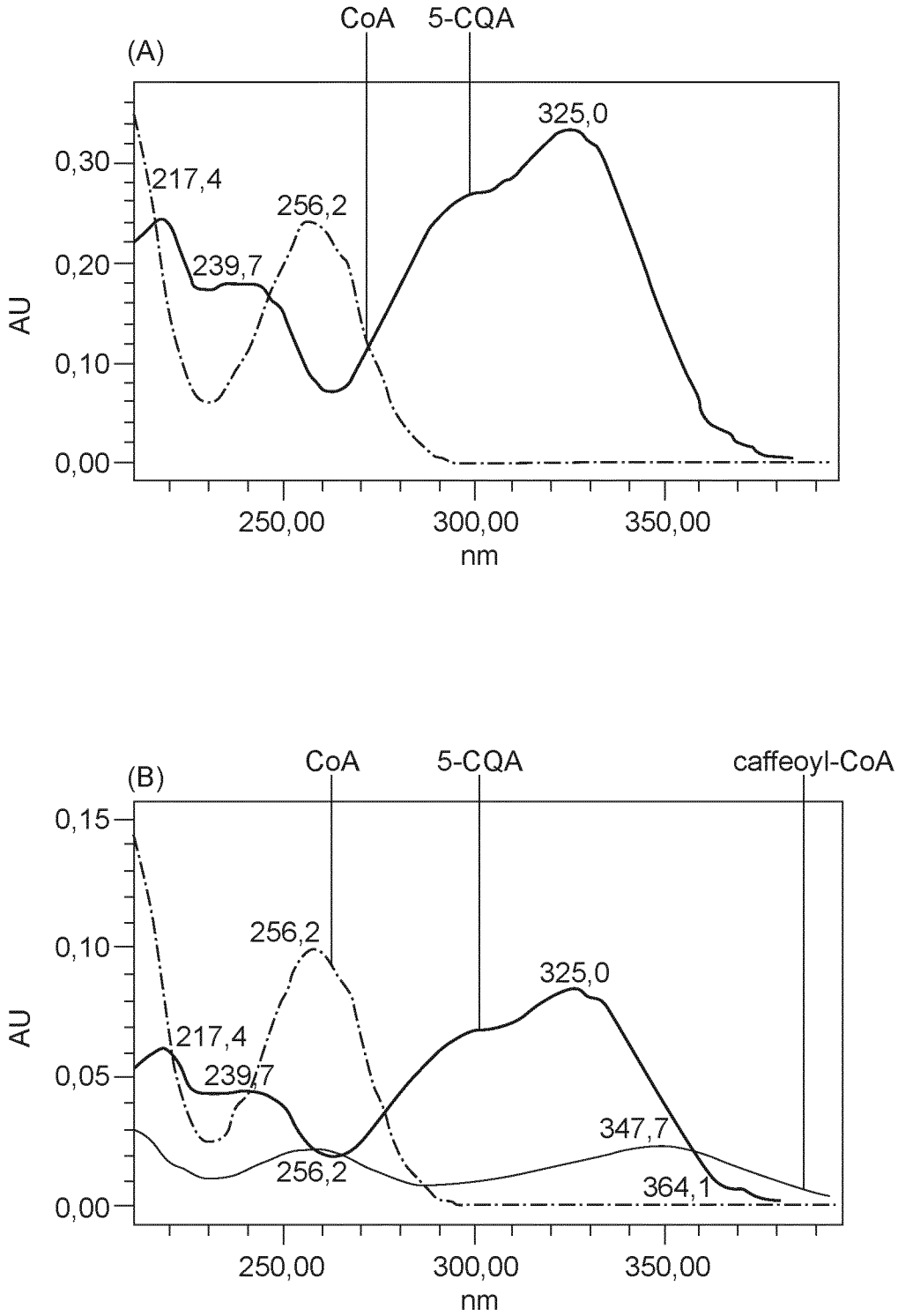

(A)
CoA
5-CQA
325,0
217,4
256,2
239,7
0,30
0,20
0,10
0,00
AU
250,00
300,00
350,00
nm
(B)
CoA
5-CQA
caffeoyl-CoA
0,15
0,10
0,05
0,00
256,2
325,0
217,4
239,7
347,7
256,2
364,1
AU
250,00
300,00
350,00
nm

FIG. 12

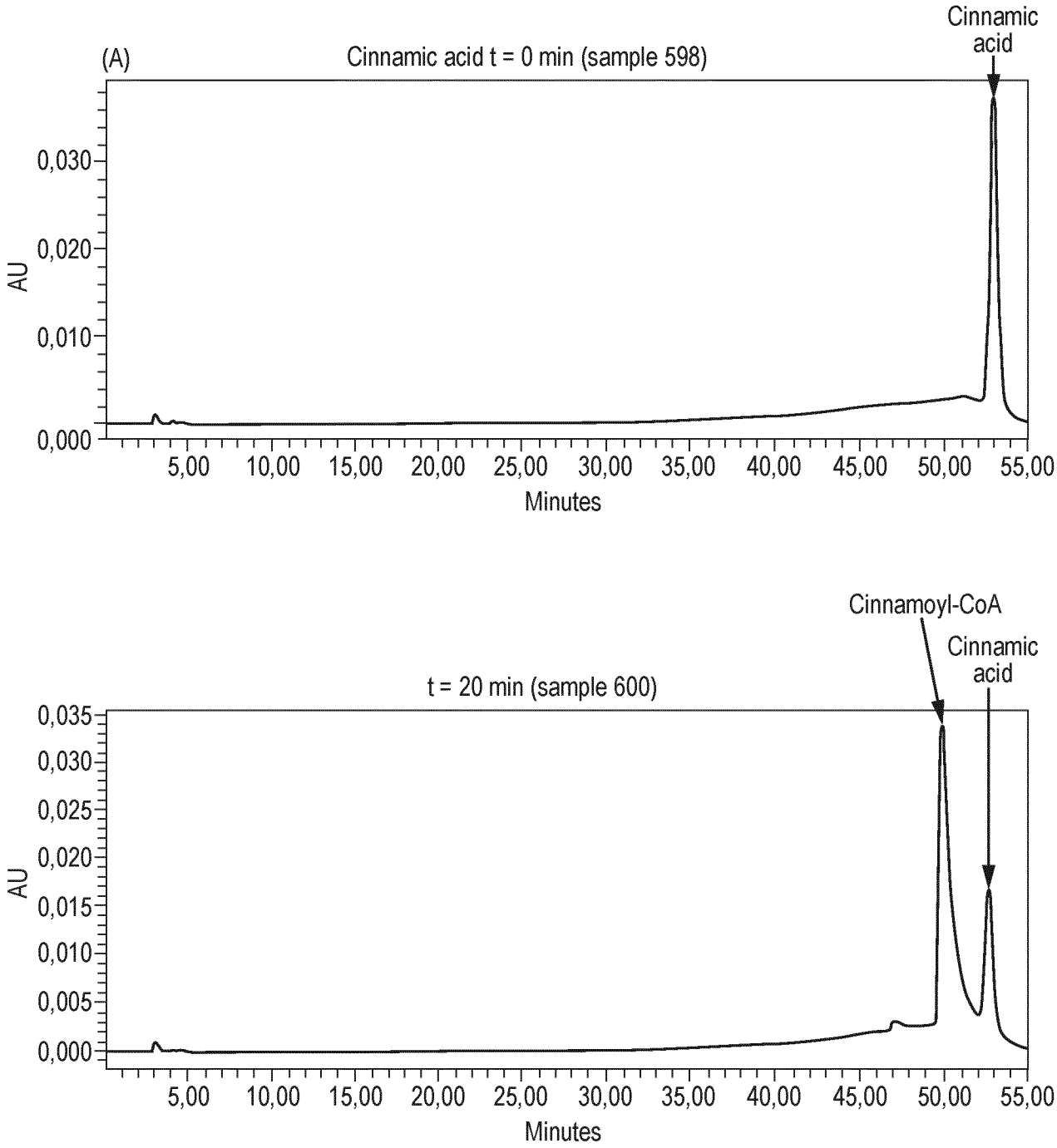

(A)
Cinnamic acid t = 0 min (sample 598)
Cinnamic acid
AU
0,030
0,020
0,010
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
Cinnamoyl-CoA
Cinnamic acid
t = 20 min (sample 600)
AU
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 13

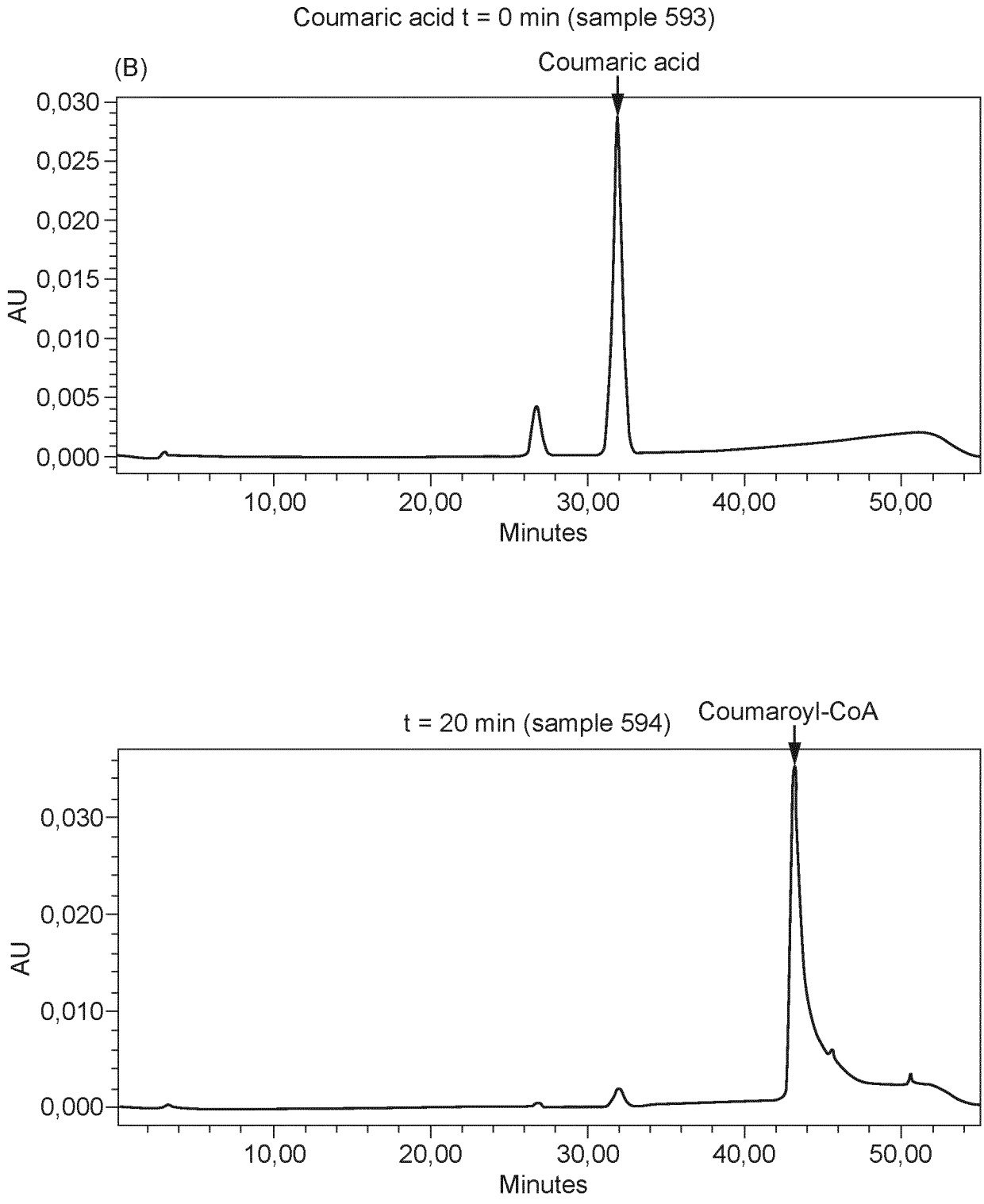

Coumaric acid t = 0 min (sample 593)
(B)
Coumaric acid
0,030
0,025
0,020
0,015
0,010
0,005
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes
t = 20 min (sample 594)
Coumaroyl-CoA
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 13 Cont'd

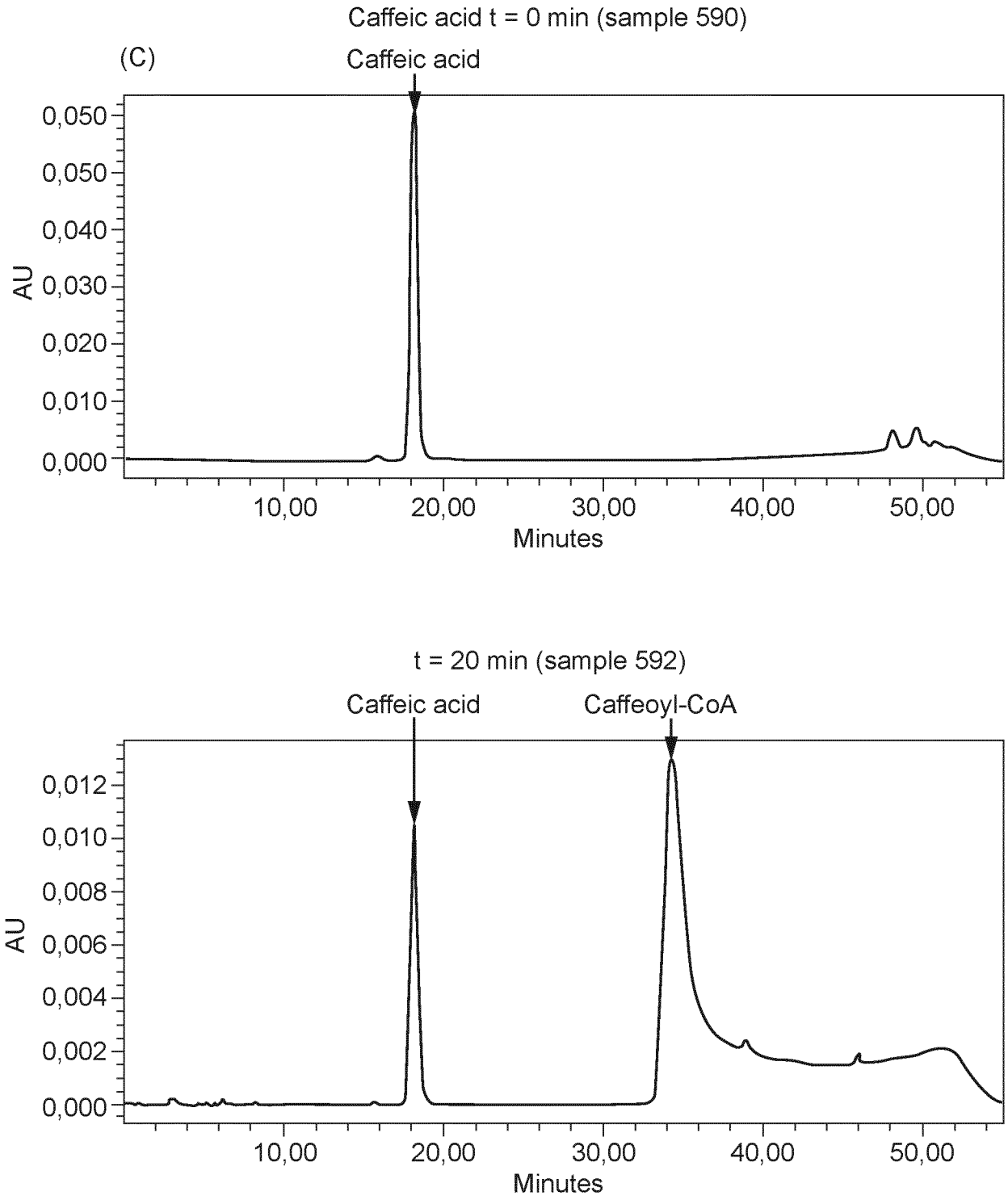

Caffeic acid t = 0 min (sample 590)
(C)
Caffeic acid
0,050
0,050
0,040
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes
t = 20 min (sample 592)
Caffeic acid
Caffeoyl-CoA
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 13 Cont'd

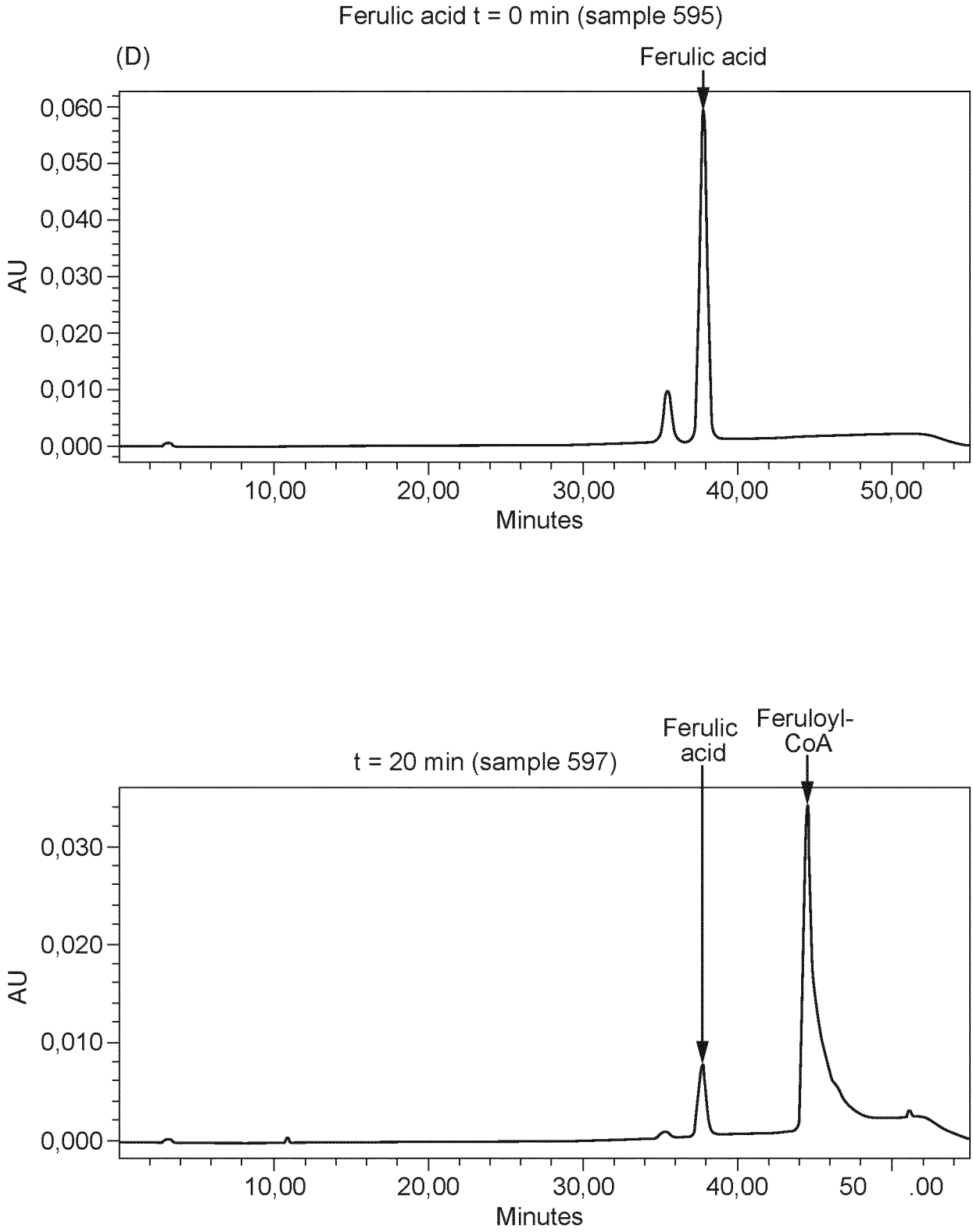

Ferulic acid t = 0 min (sample 595)
(D)
Ferulic acid
0,060
0,050
0,040
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes
t = 20 min (sample 597)
Ferulic acid
Feruloyl-CoA
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50
.00
Minutes

FIG. 13 Cont'd

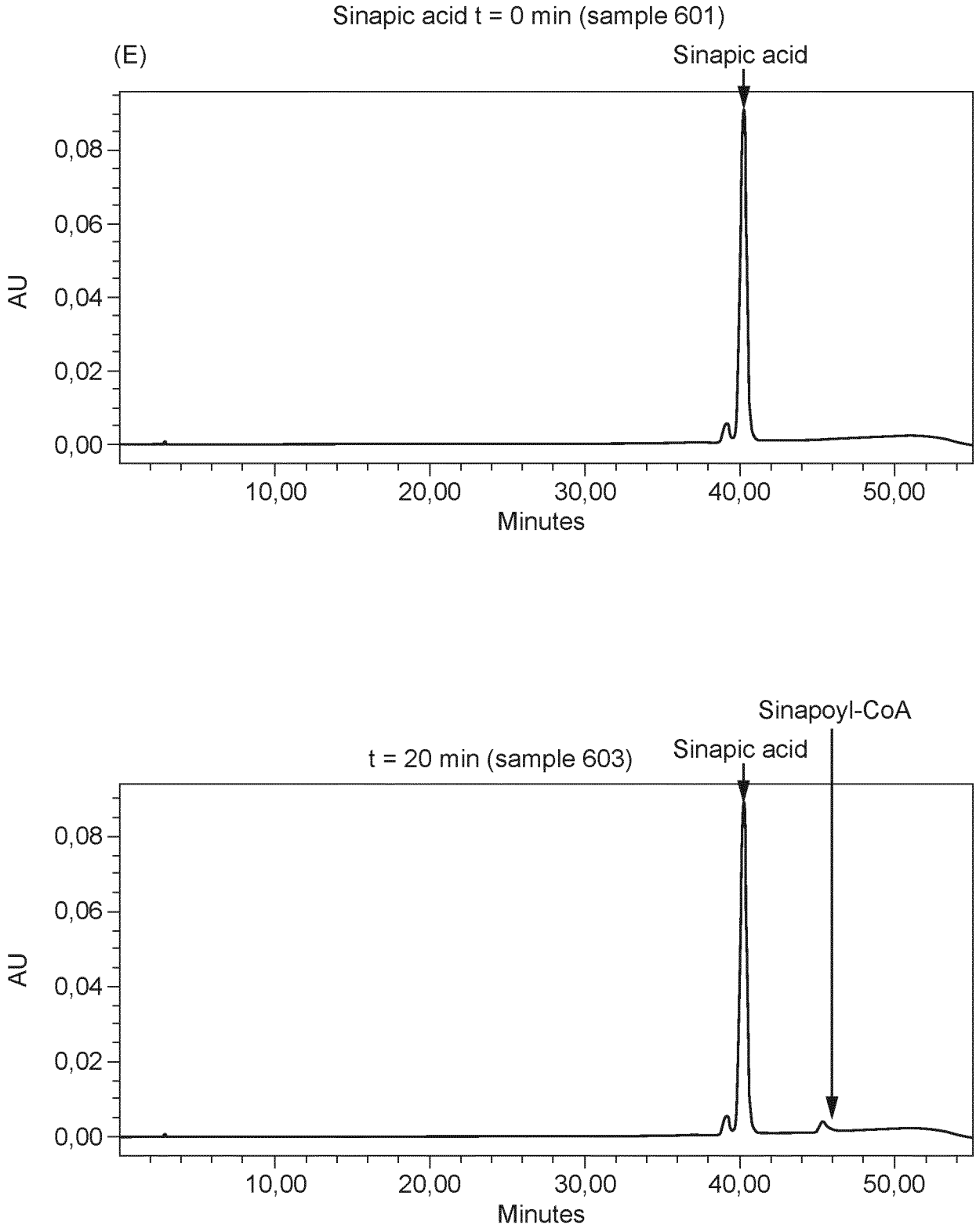

Sinapic acid t = 0 min (sample 601)
(E)
Sinapic acid
AU
0,08
0,06
0,04
0,02
0,00
10,00
20,00
30,00
40,00
50,00
Minutes
t = 20 min (sample 603)
Sinapoyl-CoA
Sinapic acid
AU
0,08
0,06
0,04
0,02
0,00
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 13 Cont'd

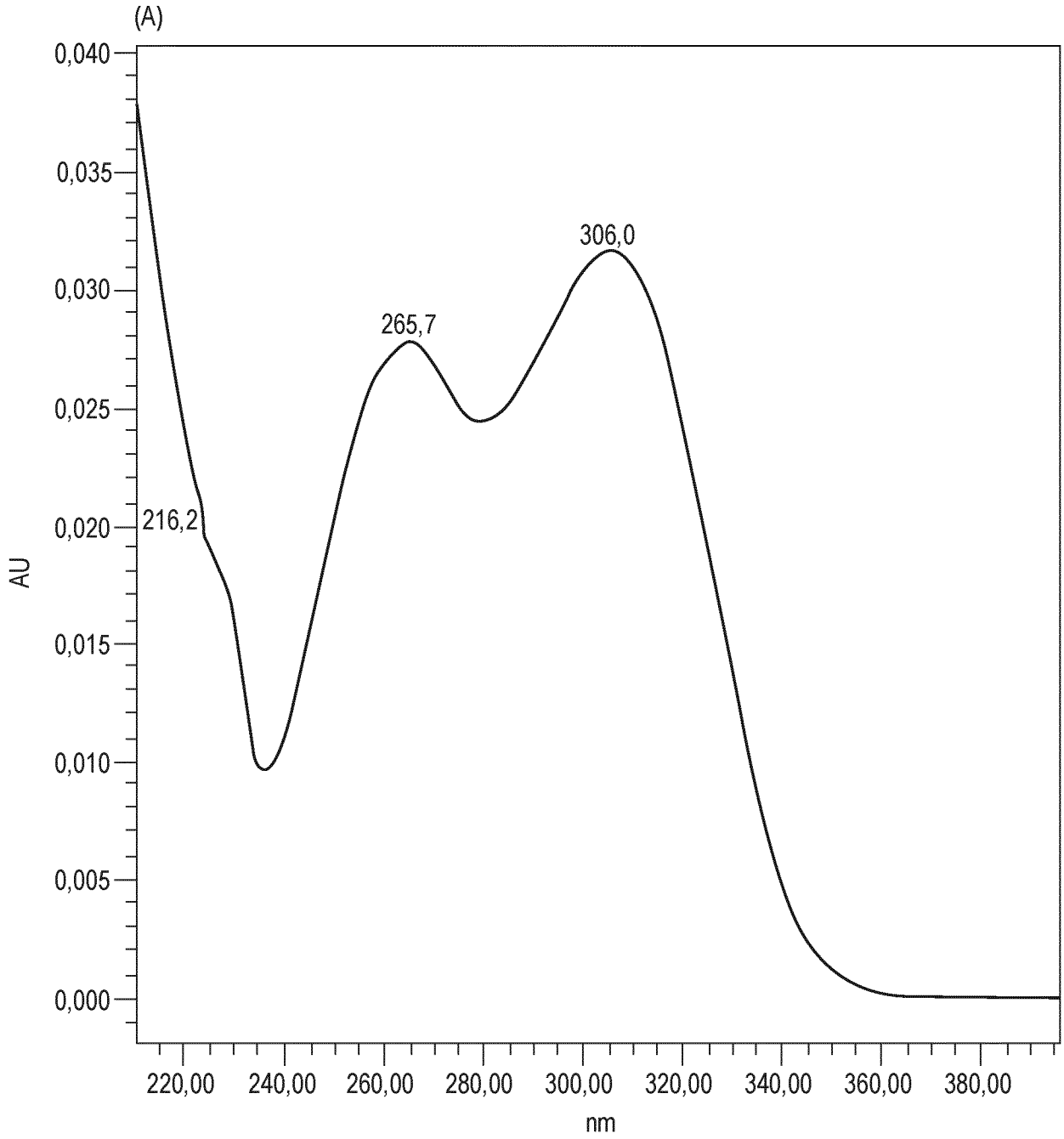

(A)
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
AU
306,0
265,7
216,2
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 14

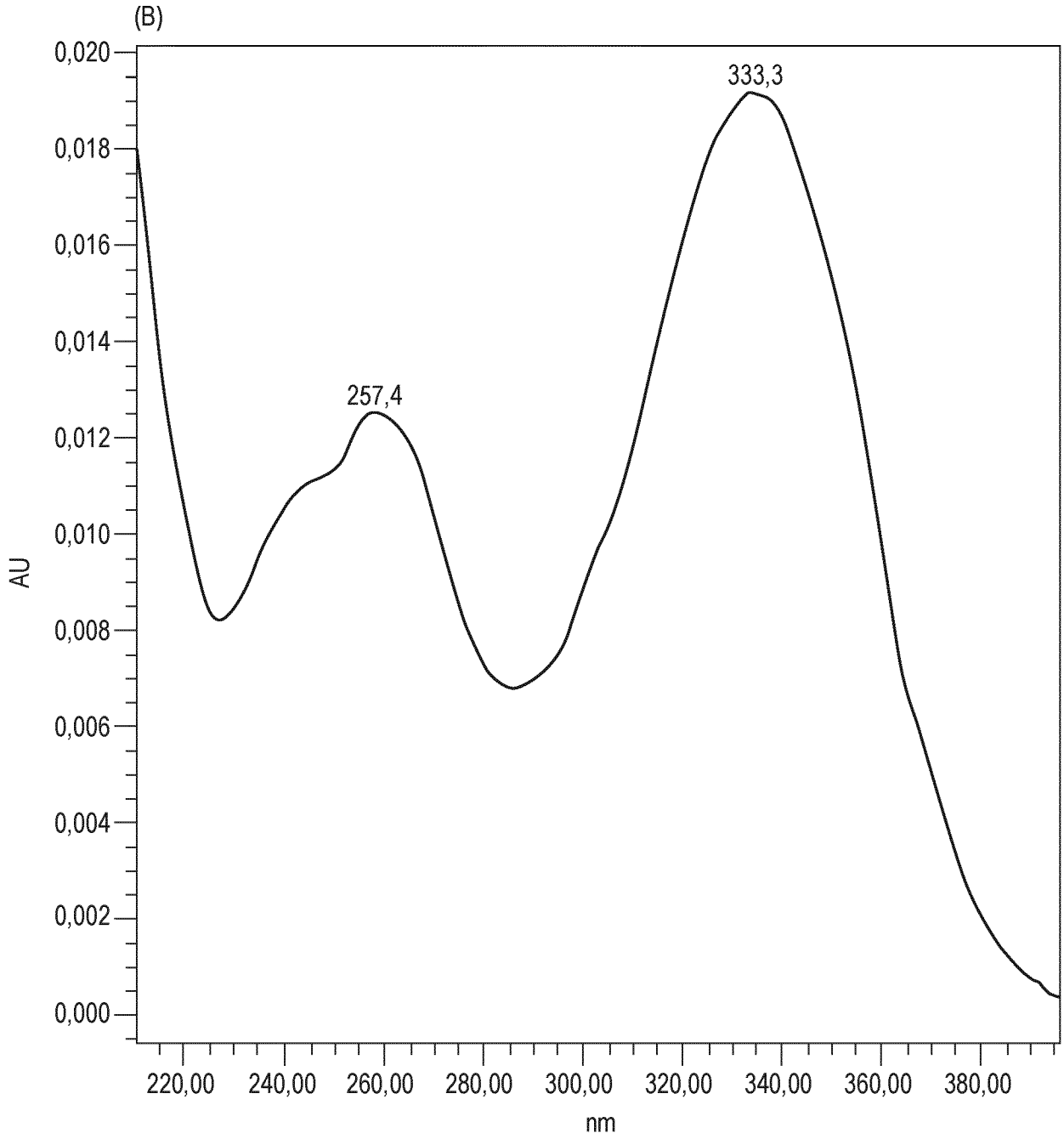

(B)
0,020
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
333,3
257,4
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 14 Cont'd

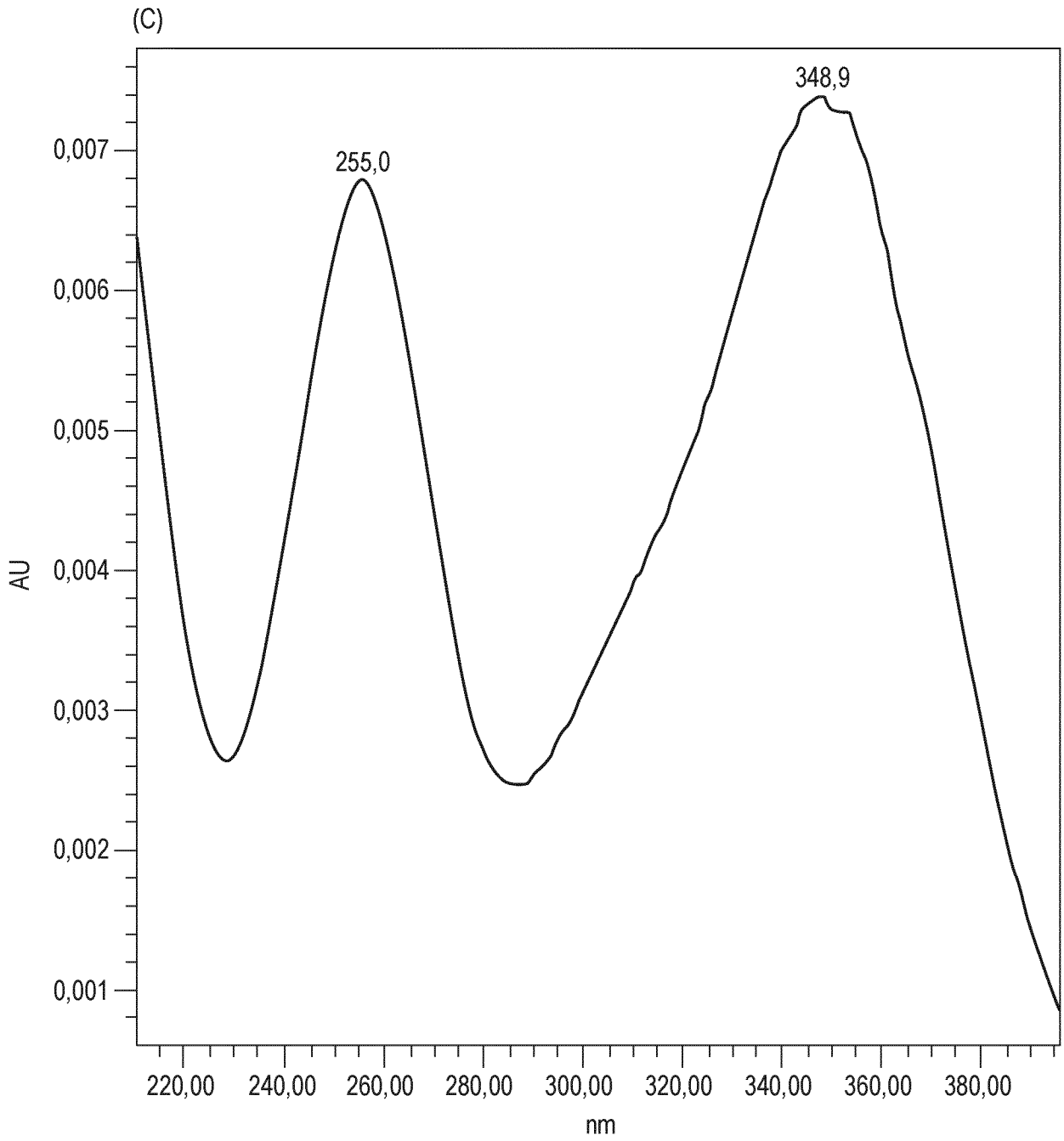

(C)
348,9
255,0
0,007
0,006
0,005
0,004
0,003
0,002
0,001
AU
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 14 Cont'd

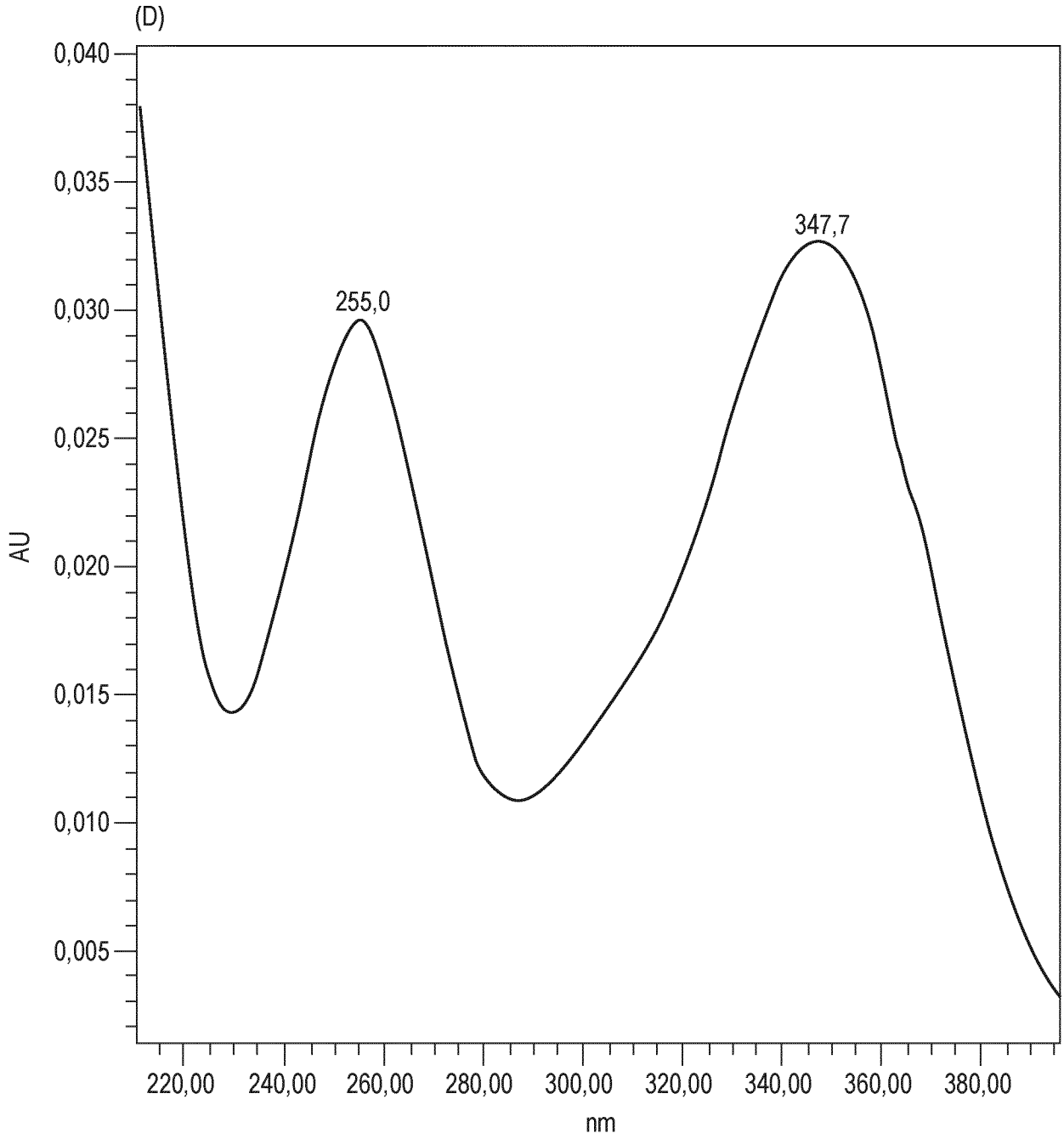

(D)
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
AU
255,0
347,7
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 14 Cont'd

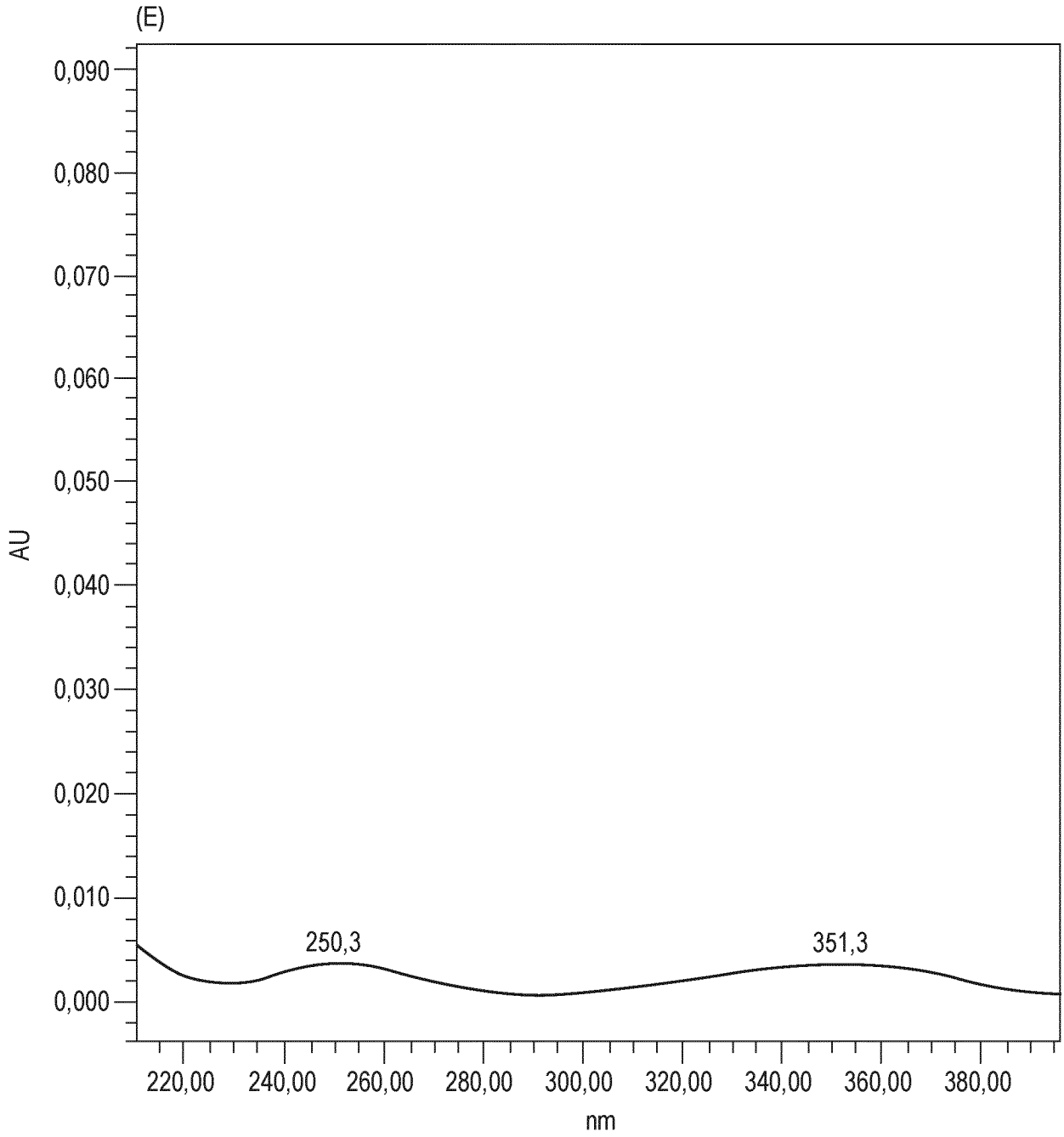

(E)
0,090
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
AU
250,3
351,3
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 14 Cont'd

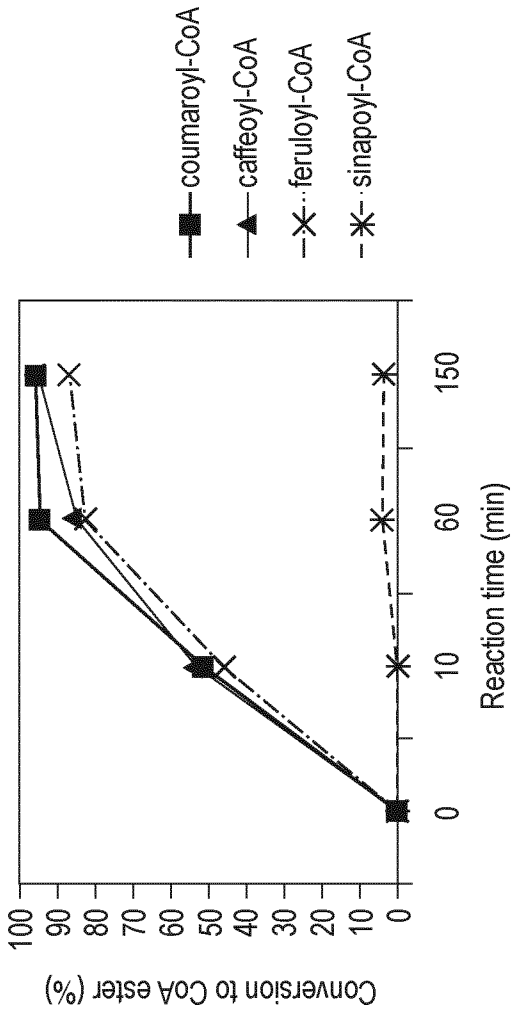

coumaroyl-CoA
caffeoyl-CoA
feruloyl-CoA
sinapoyl-CoA
Conversion to CoA ester (%)
0
10
20
30
40
50
60
70
80
90
100
0
10
60
150
Reaction time (min)

FIG. 15

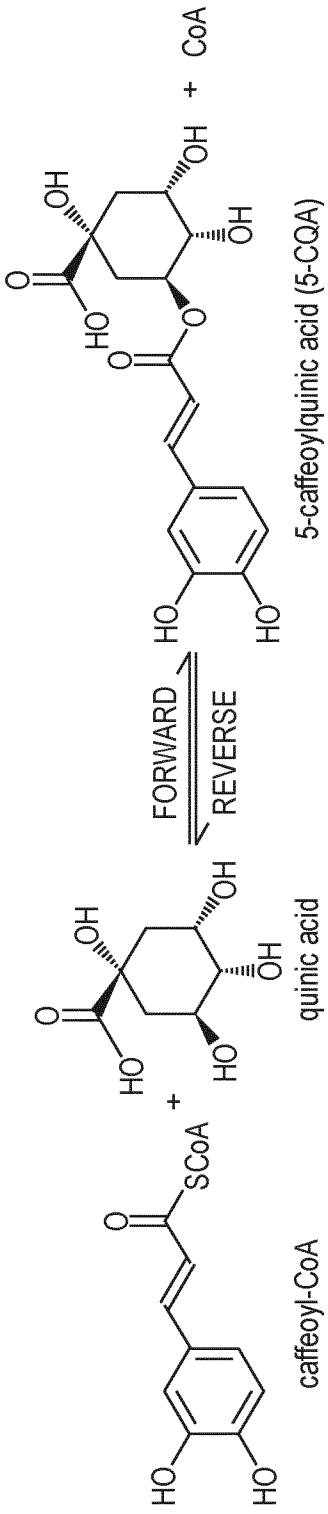

caffeoyl-CoA
quinic acid
FORWARD
REVERSE
5-caffeoylquinic acid (5-CQA)
CoA

FIG. 16

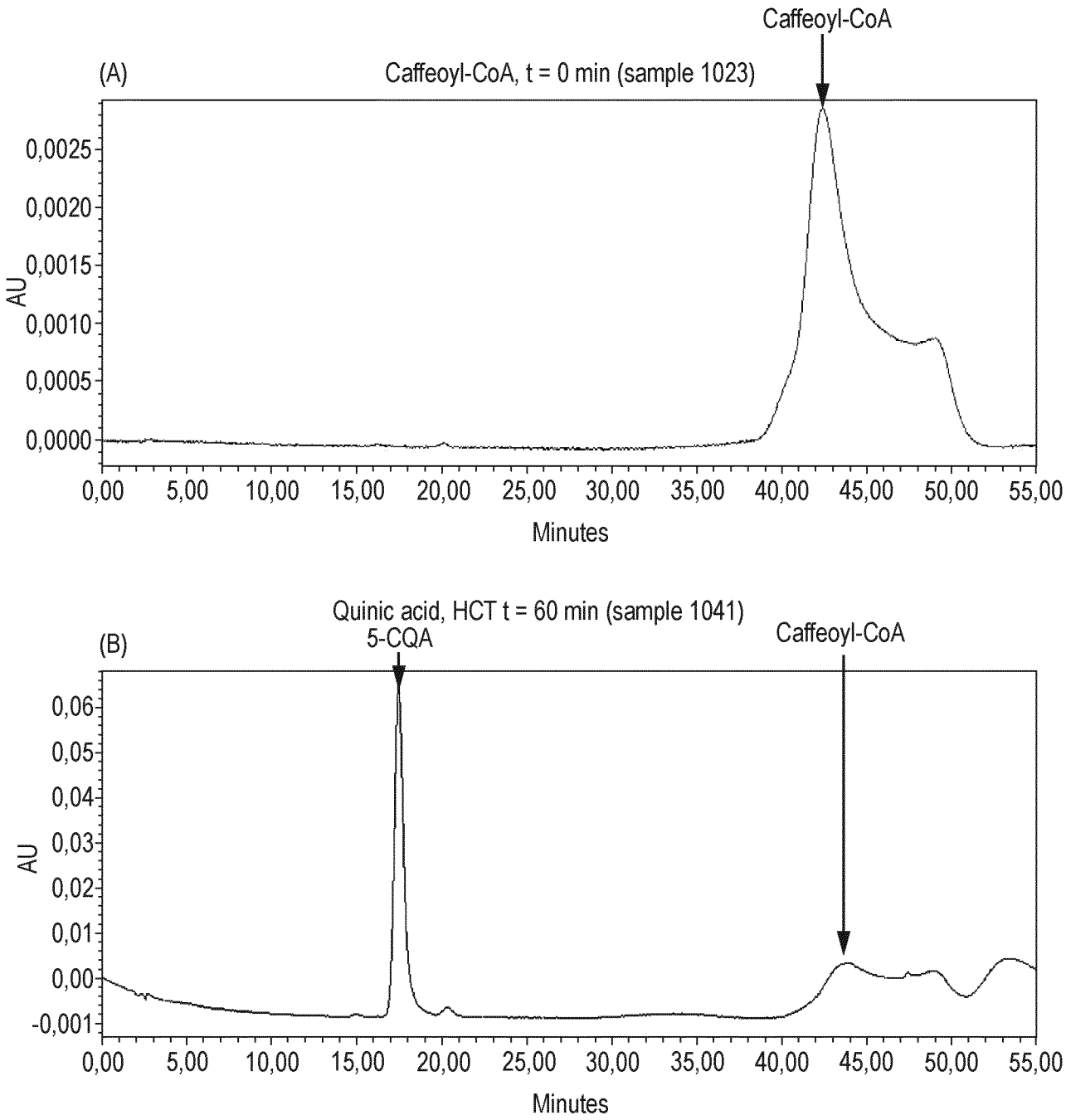

(A)
Caffeoyl-CoA, t = 0 min (sample 1023)
Caffeoyl-CoA
AU
0,0025
0,0020
0,0015
0,0010
0,0005
0,0000
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
(B)
Quinic acid, HCT t = 60 min (sample 1041)
5-CQA
Caffeoyl-CoA
AU
0,06
0,05
0,04
0,03
0,02
0,01
0,00
-0,001
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 17

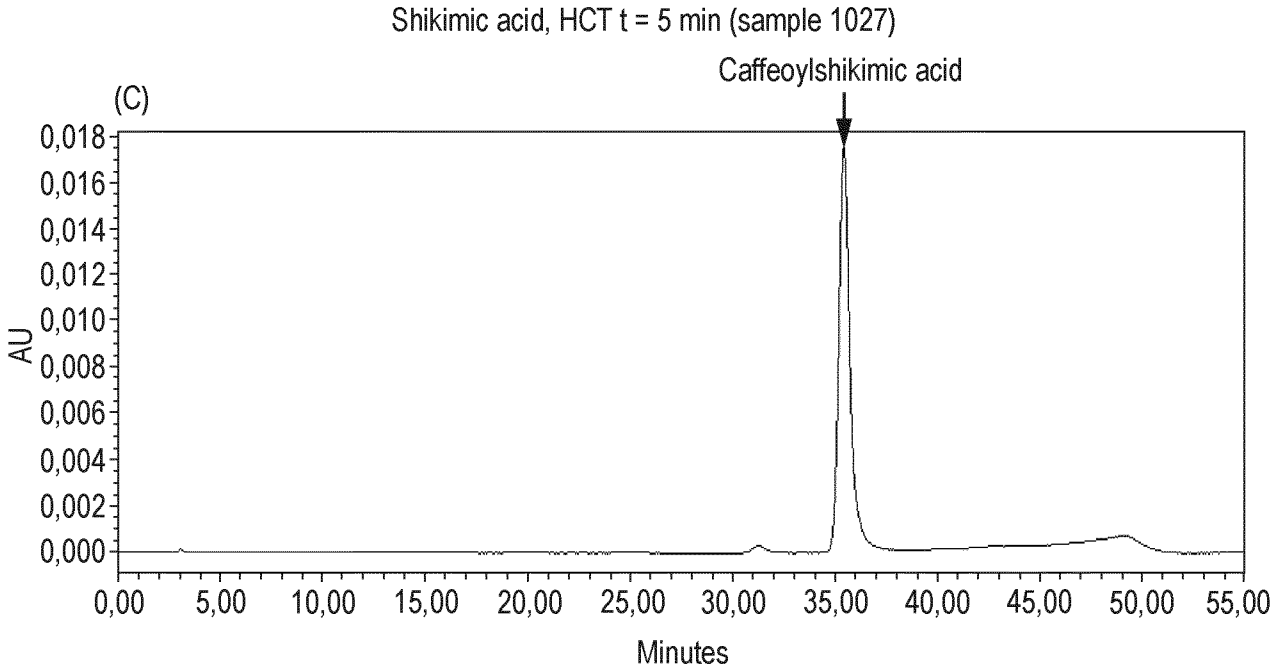

Shikimic acid, HCT t = 5 min (sample 1027)
Caffeoylshikimic acid
(C)
AU
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 17 Cont'd

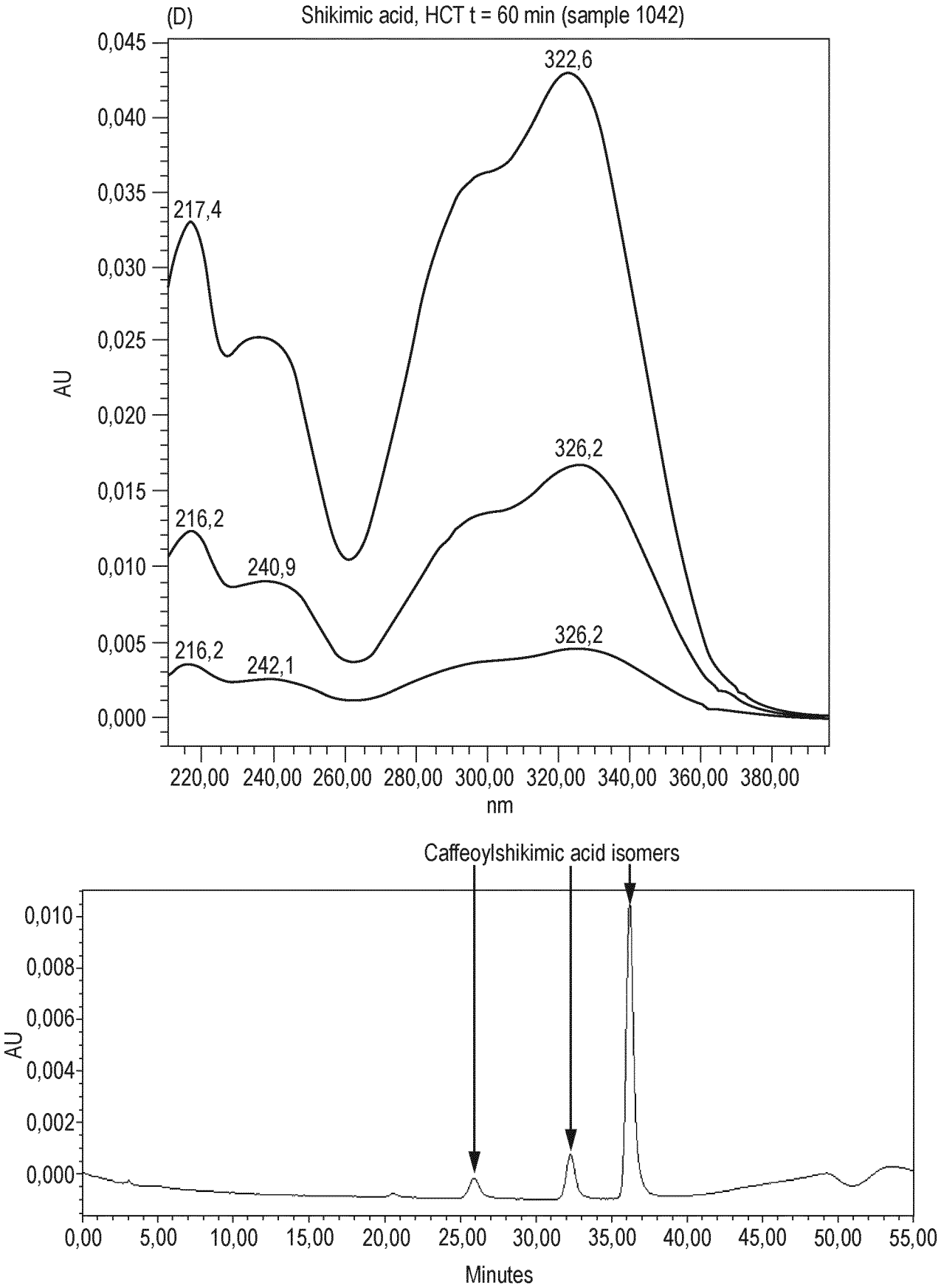
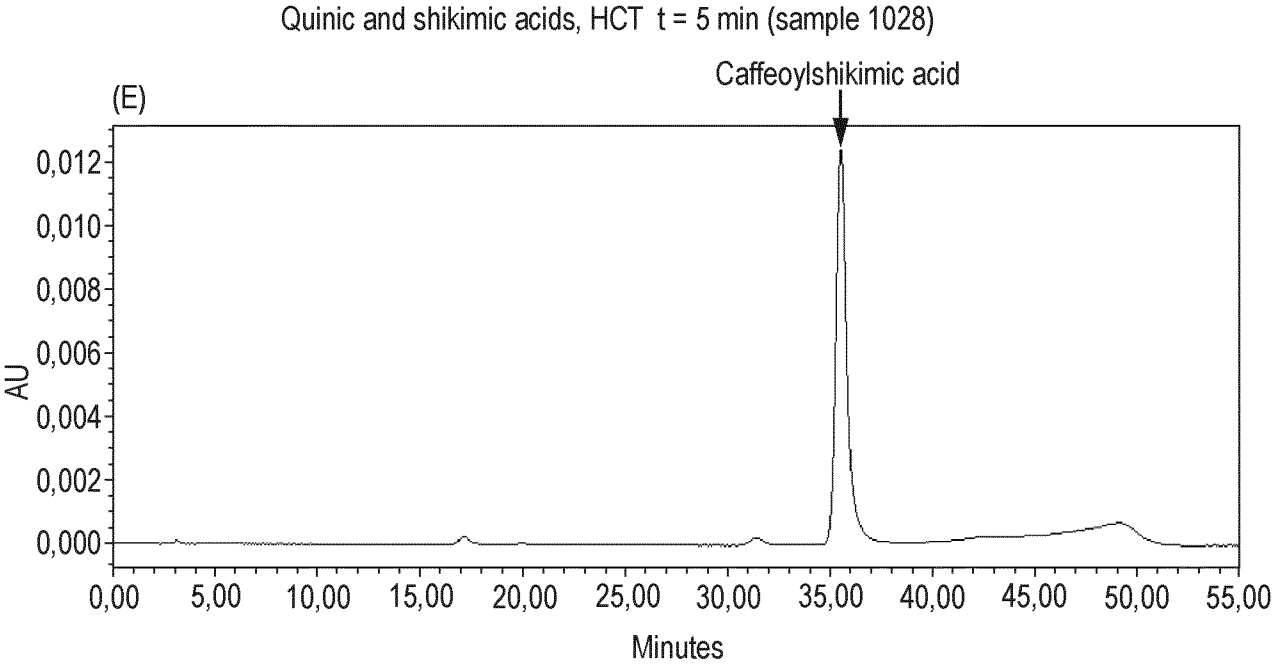

(D)
Shikimic acid, HCT t = 60 min (sample 1042)
0,045
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
AU
322,6
217,4
326,2
216,2
240,9
326,2
216,2
242,1
220,00 240,00 260,00 280,00 300,00 320,00 340,00 360,00 380,00
nm
Caffeoylshikimic acid isomers
0,010
0,008
0,006
0,004
0,002
0,000
AU
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 17 Cont'd


Quinic and shikimic acids, HCT t = 5 min (sample 1028)
(E)
Caffeoylshikimic acid
AU
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

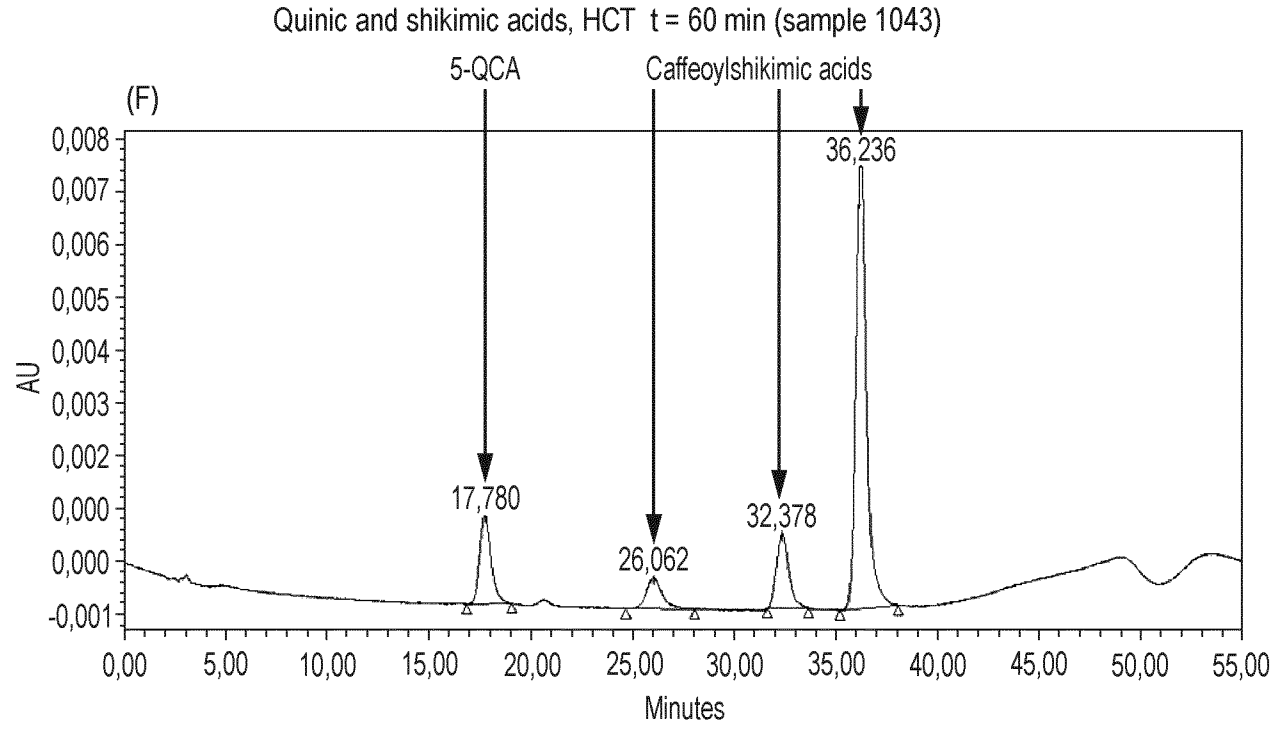

Quinic and shikimic acids, HCT t = 60 min (sample 1043)
(F)
5-QCA
Caffeoylshikimic acids
36,236
17,780
26,062
32,378
AU
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,000
0,000
-0,001
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 17 Cont'd

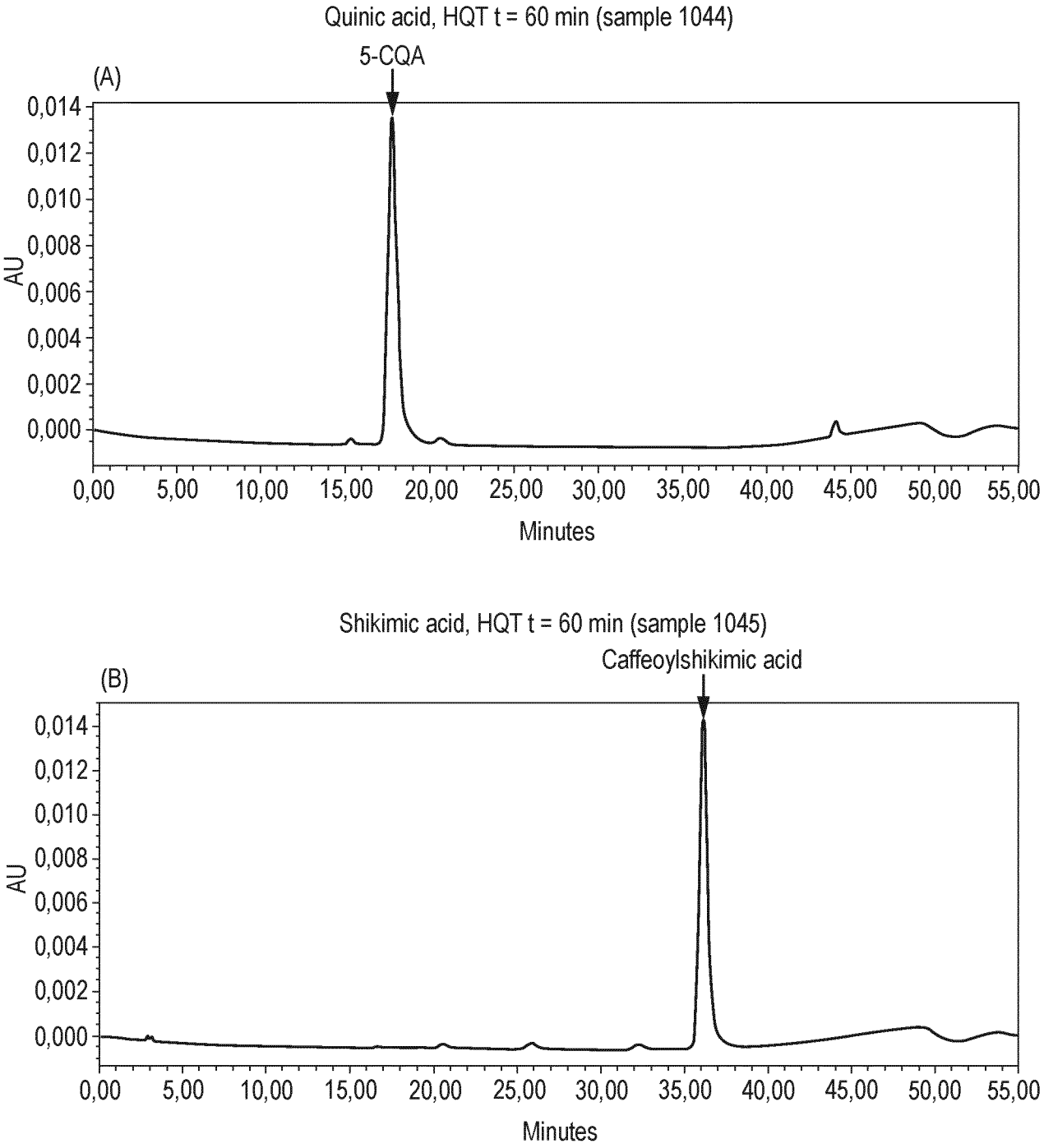
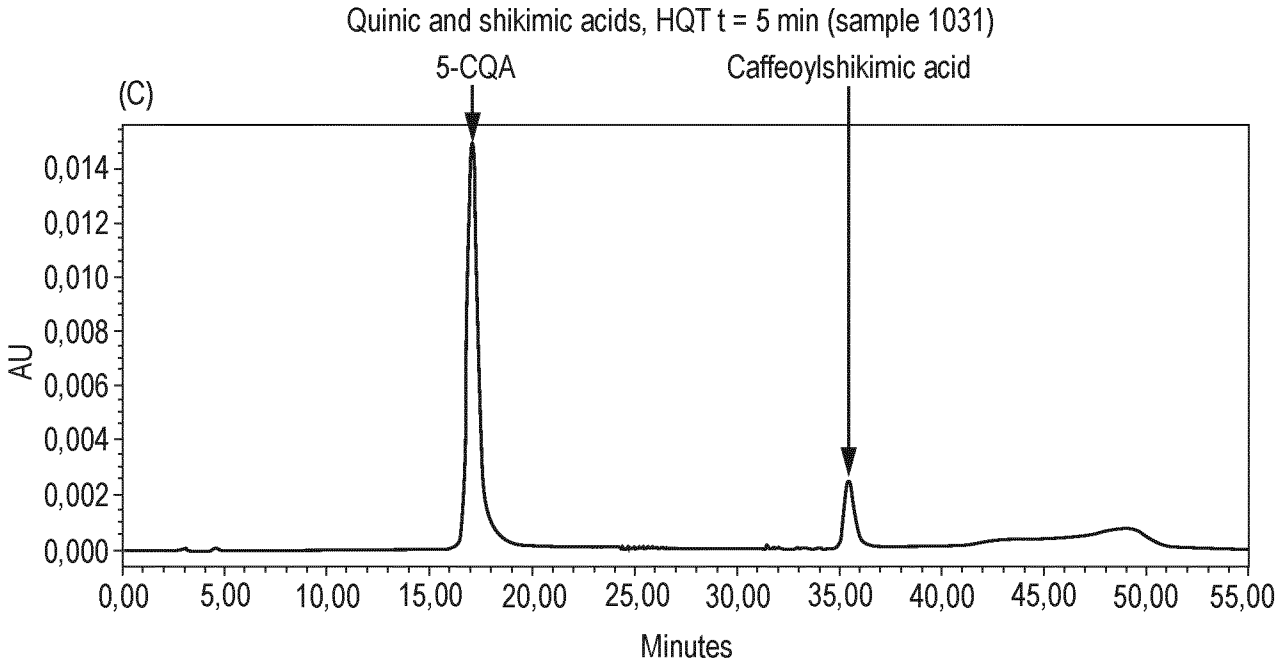

Quinic acid, HQT t = 60 min (sample 1044)
5-CQA
(A)
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
Shikimic acid, HQT t = 60 min (sample 1045)
Caffeoylshikimic acid
(B)
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 18


Quinic and shikimic acids, HQT t = 5 min (sample 1031)
(C)
5-CQA
Caffeoylshikimic acid
AU
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

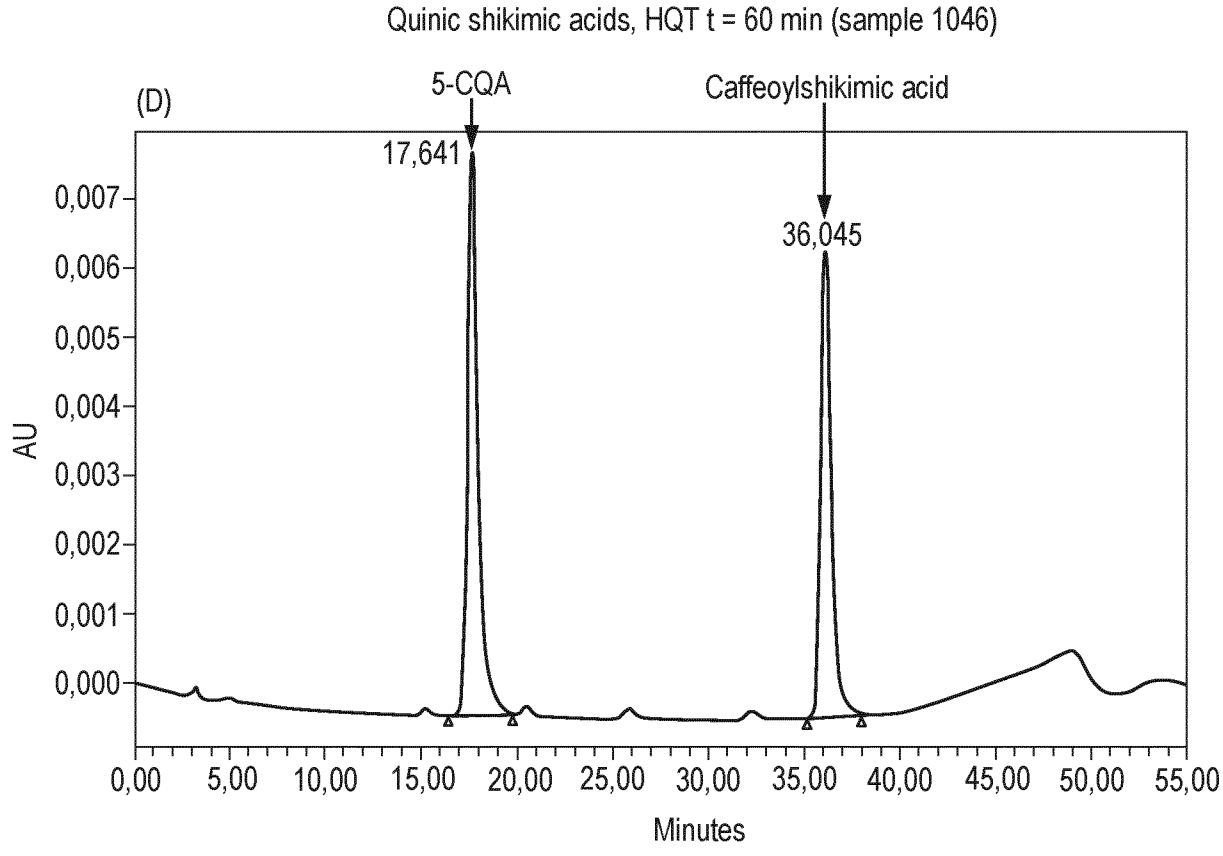

Quinic shikimic acids, HQT t = 60 min (sample 1046)
(D)
5-CQA
Caffeoylshikimic acid
17,641
36,045
AU
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 18 Cont'd

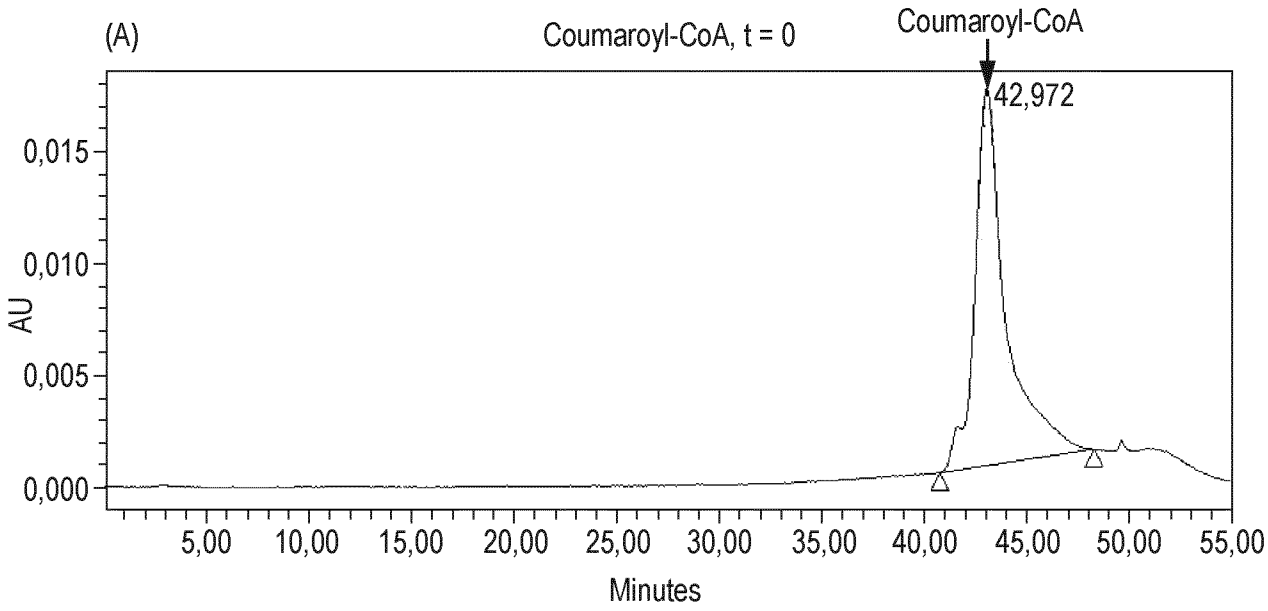

(A)
Coumaroyl-CoA, t = 0
Coumaroyl-CoA
42,972
0,015
0,010
0,005
0,000
AU
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 19

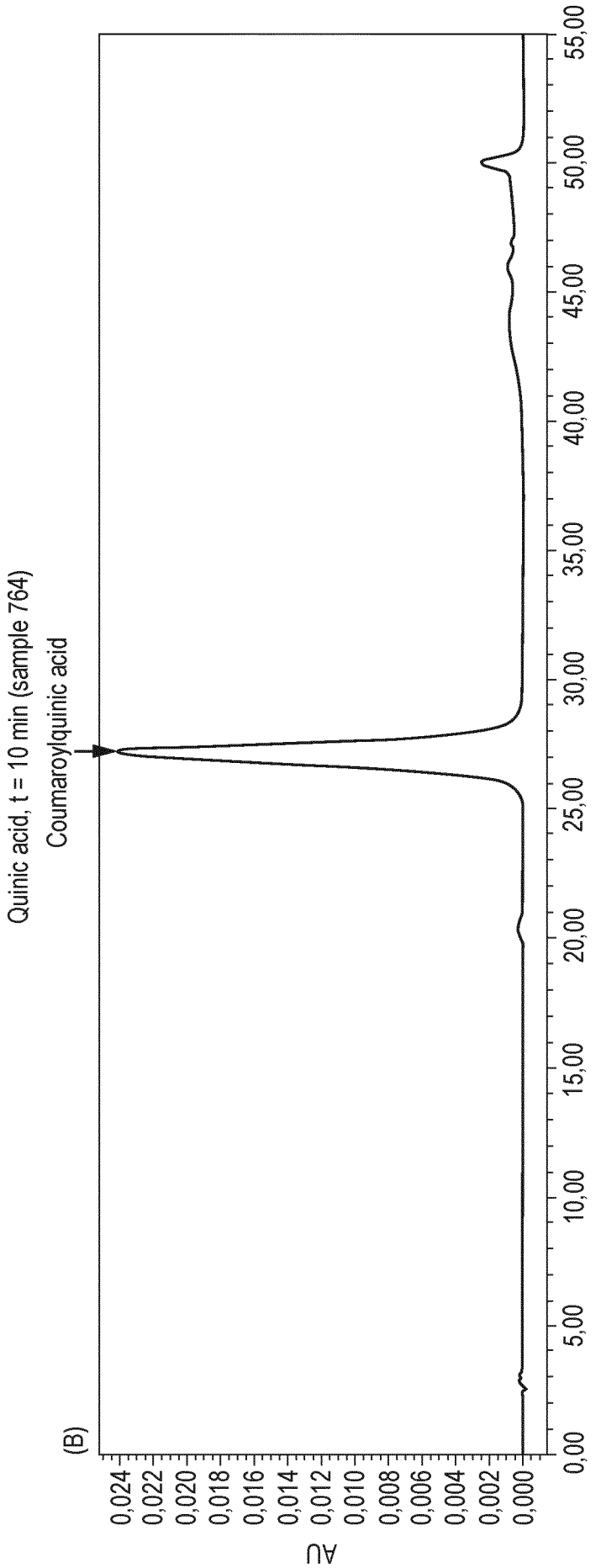

(B)
Quinic acid, t = 10 min (sample 764)
Coumaroylquinic acid
AU
0,024
0,022
0,020
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 19 Cont'd

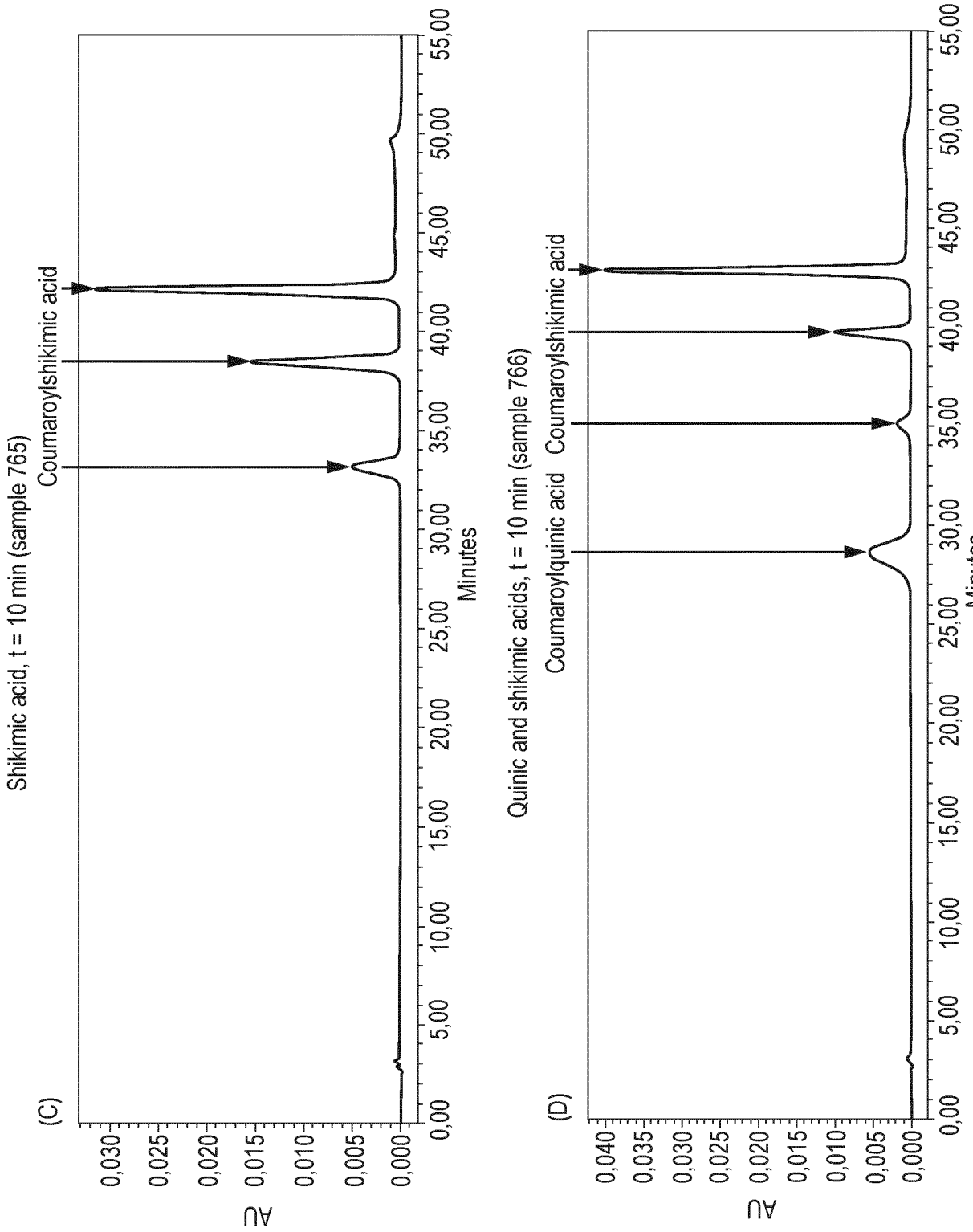

(C)
Shikimic acid, t = 10 min (sample 765)
Coumaroylshikimic acid
AU
0,030
0,025
0,020
0,015
0,010
0,005
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
(D)
Quinic and shikimic acids, t = 10 min (sample 766)
Coumaroylquinic acid
Coumaroylshikimic acid
AU
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 19 Cont'd

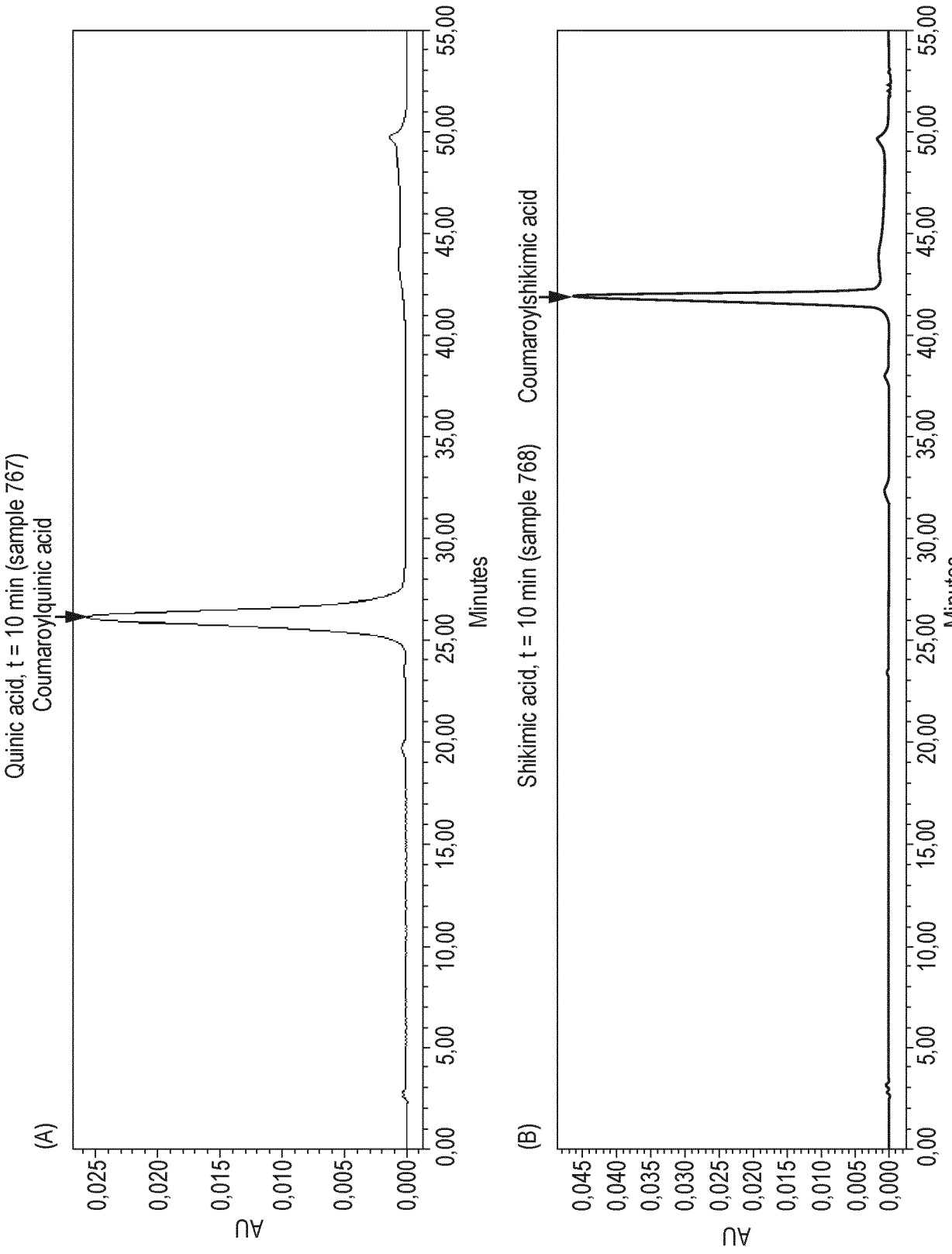

(A)
Quinic acid, t = 10 min (sample 767)
Coumaroylquinic acid
AU
0,025
0,020
0,015
0,010
0,005
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
(B)
Shikimic acid, t = 10 min (sample 768)
Coumaroylshikimic acid
AU
0,045
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
Minutes

FIG. 20

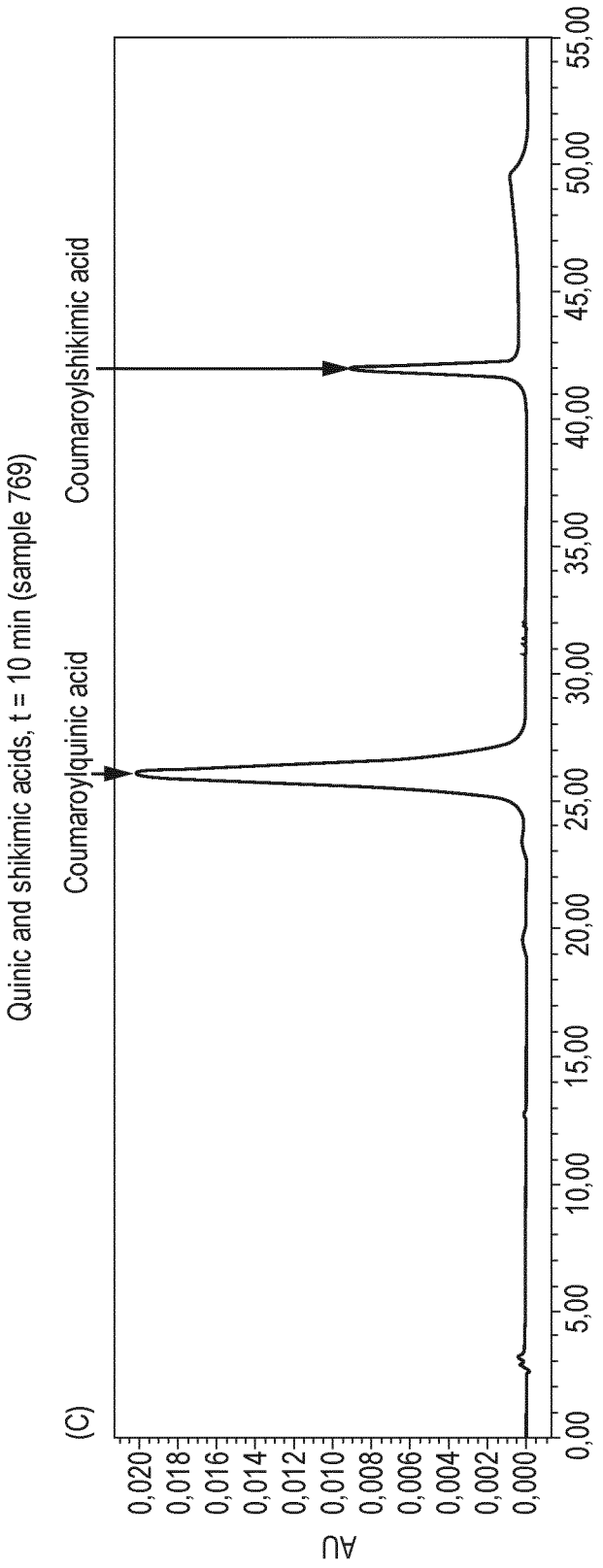

(C)
Quinic and shikimic acids, t = 10 min (sample 769)
Coumaroylquinic acid
Coumaroylshikimic acid
AU
0,000
0,002
0,004
0,006
0,008
0,010
0,012
0,014
0,016
0,018
0,020
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 20 Cont'd

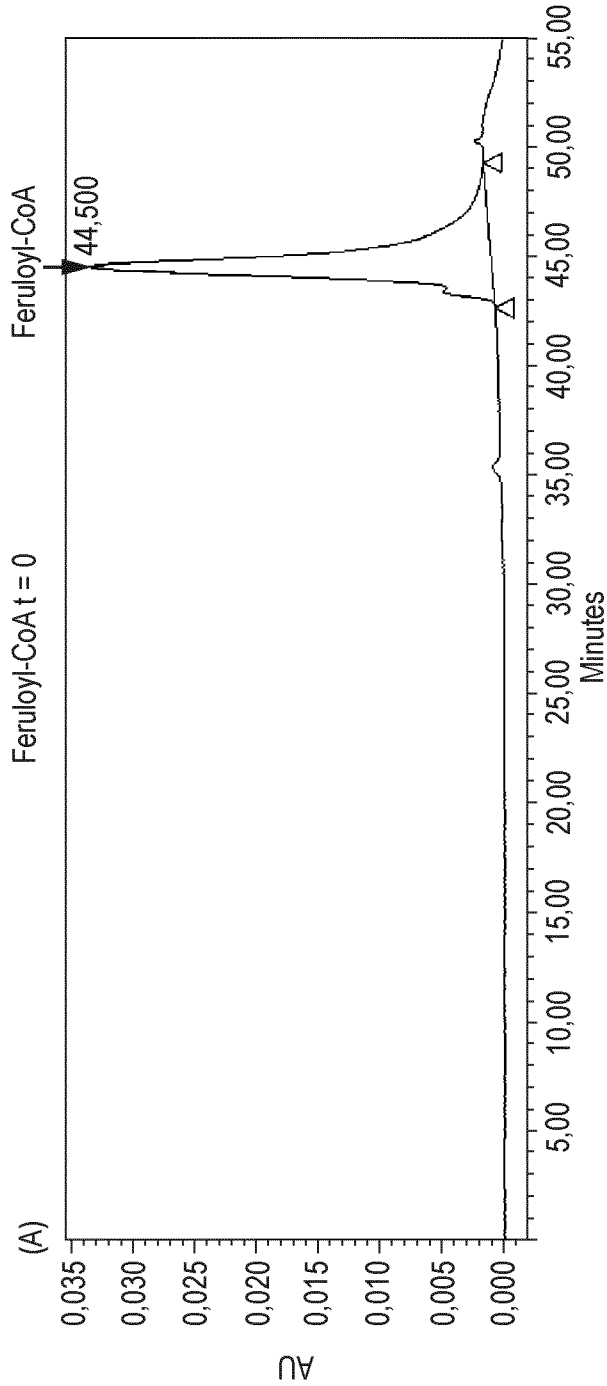
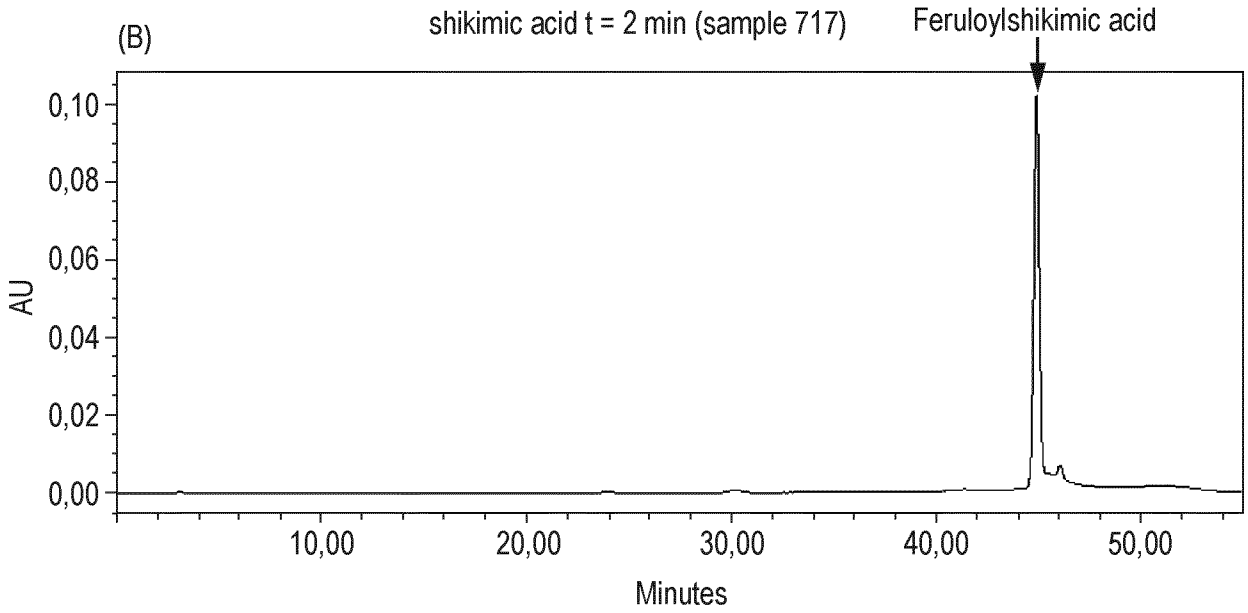

(A)
Feruloyl-CoA t = 0
Feruloyl-CoA
44,500
AU
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 21


(B)
shikimic acid t = 2 min (sample 717)
Feruloylshikimic acid
0,10
0,08
0,06
0,04
0,02
0,00
AU
10,00
20,00
30,00
40,00
50,00
Minutes

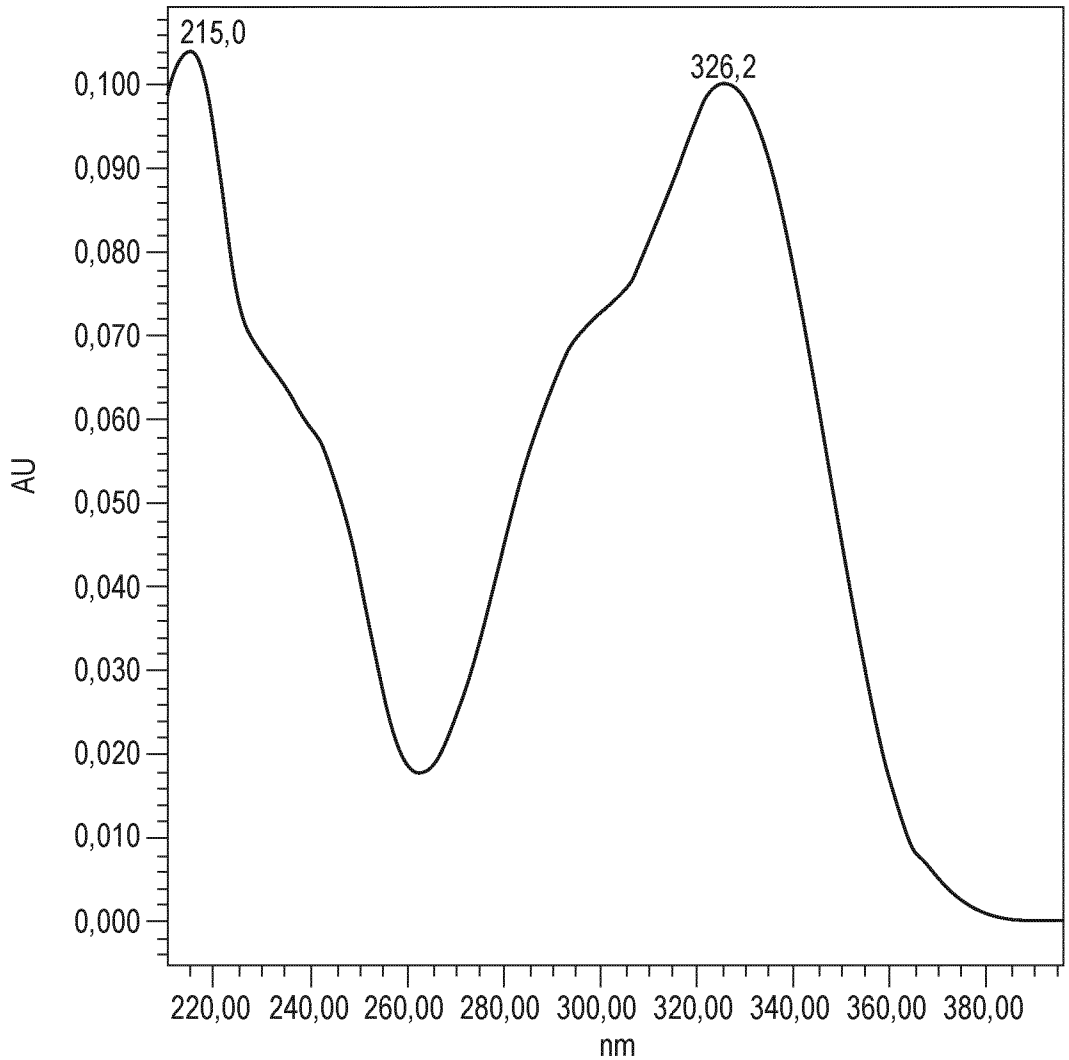

215,0
326,2
0,100
0,090
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
AU
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 21 Cont'd

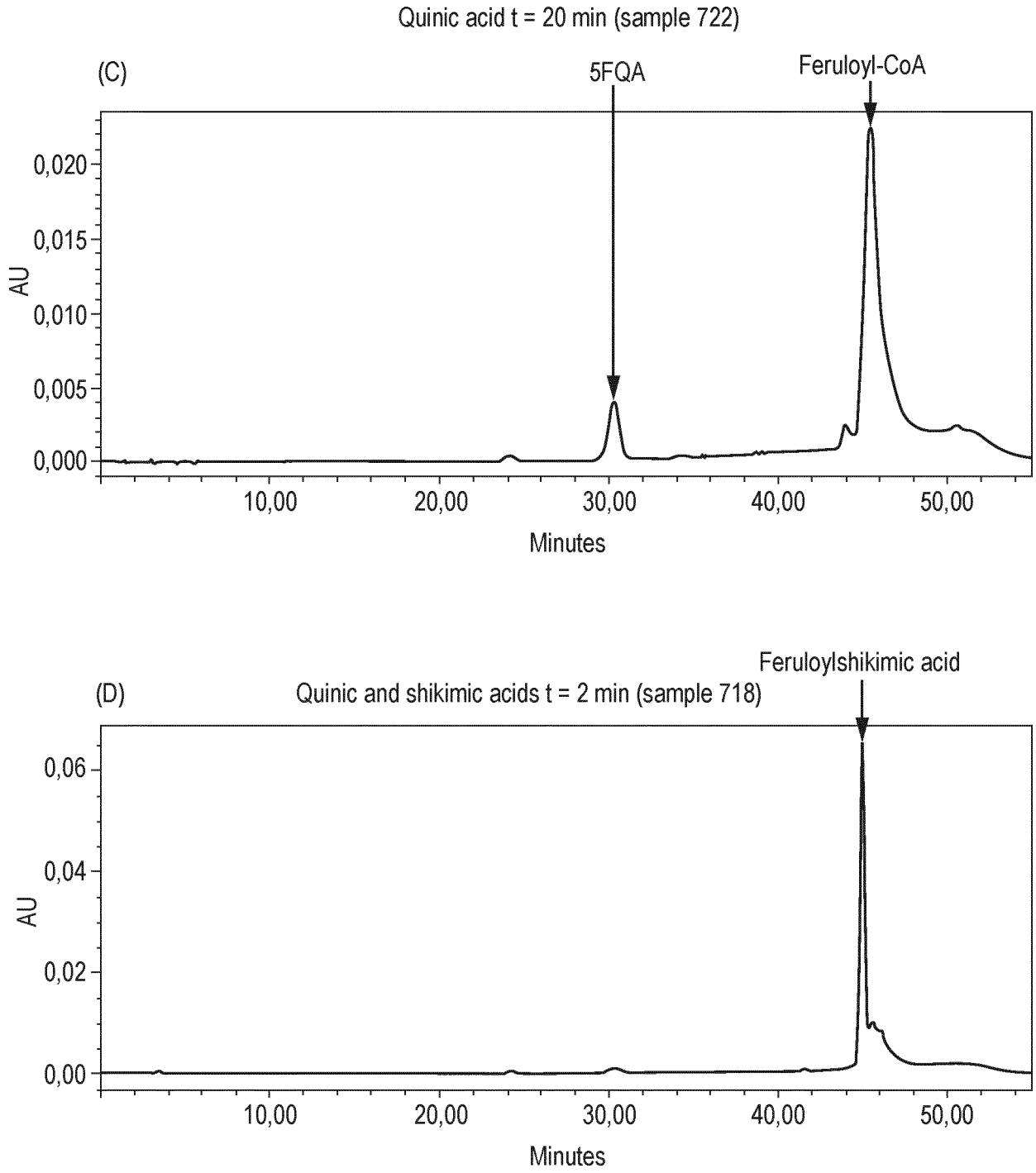
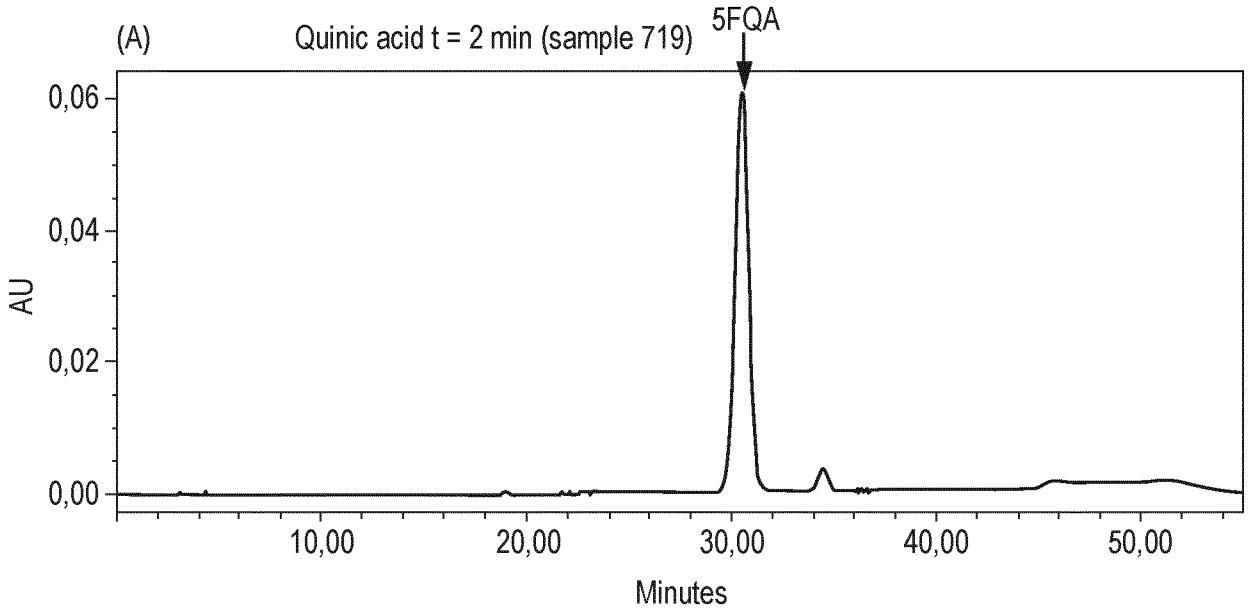

Quinic acid t = 20 min (sample 722)
(C)
5FQA
Feruloyl-CoA
0,020
0,015
0,010
0,005
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes
Feruloylshikimic acid
(D)
Quinic and shikimic acids t = 2 min (sample 718)
0,06
0,04
0,02
0,00
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 21 Cont'd


(A)
Quinic acid t = 2 min (sample 719)
5FQA
0,06
0,04
0,02
0,00
AU
10,00
20,00
30,00
40,00
50,00
Minutes

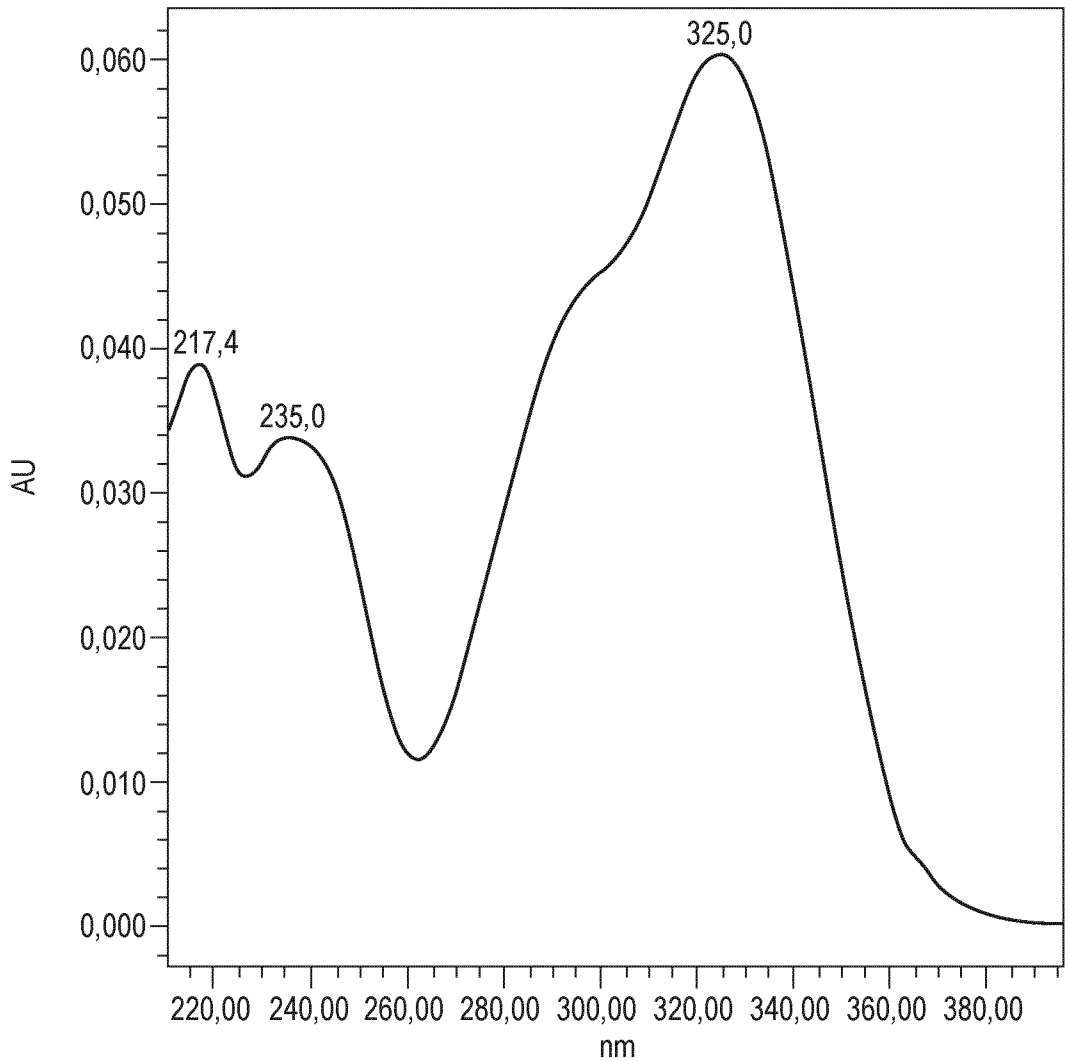
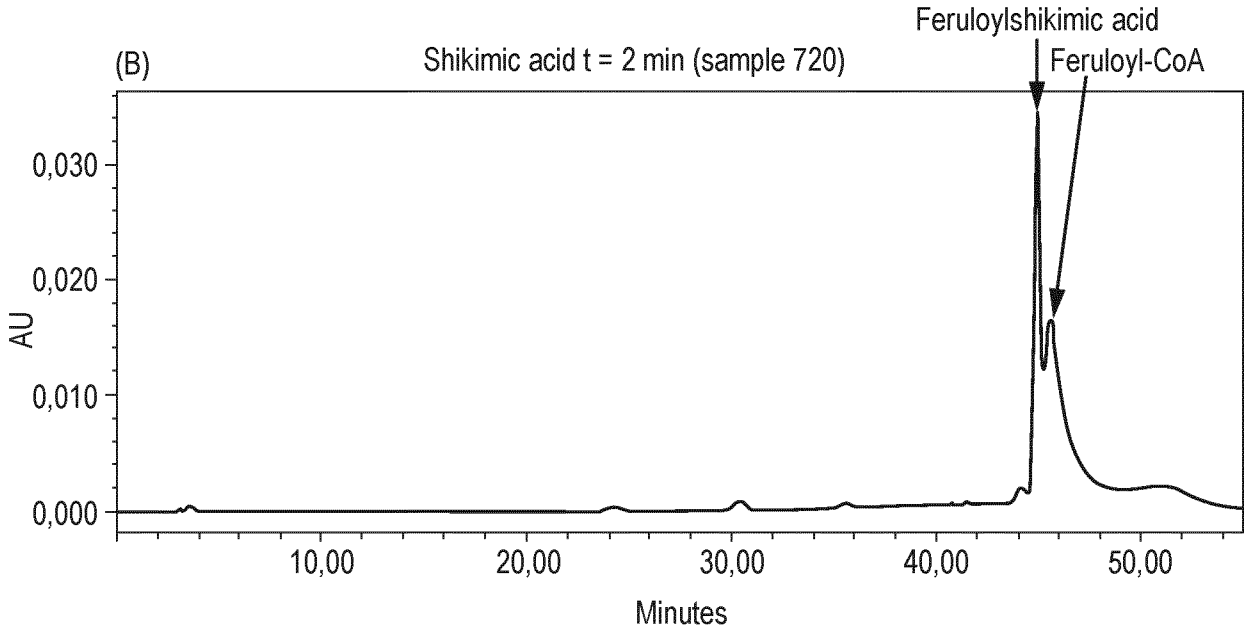

325,0
217,4
235,0
0,060
0,050
0,040
0,030
0,020
0,010
0,000
AU
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 22


(B)
Shikimic acid t = 2 min (sample 720)
Feruloylshikimic acid
Feruloyl-CoA
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

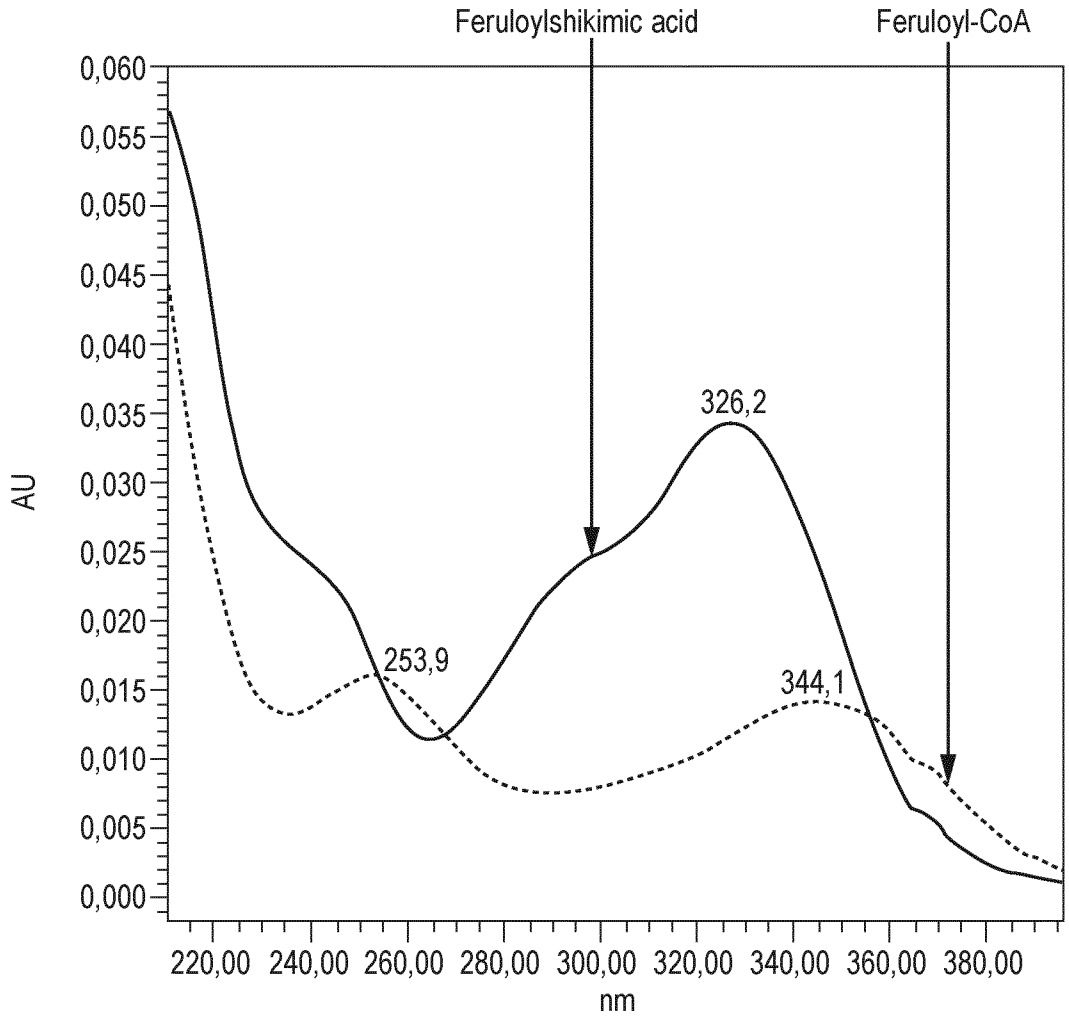
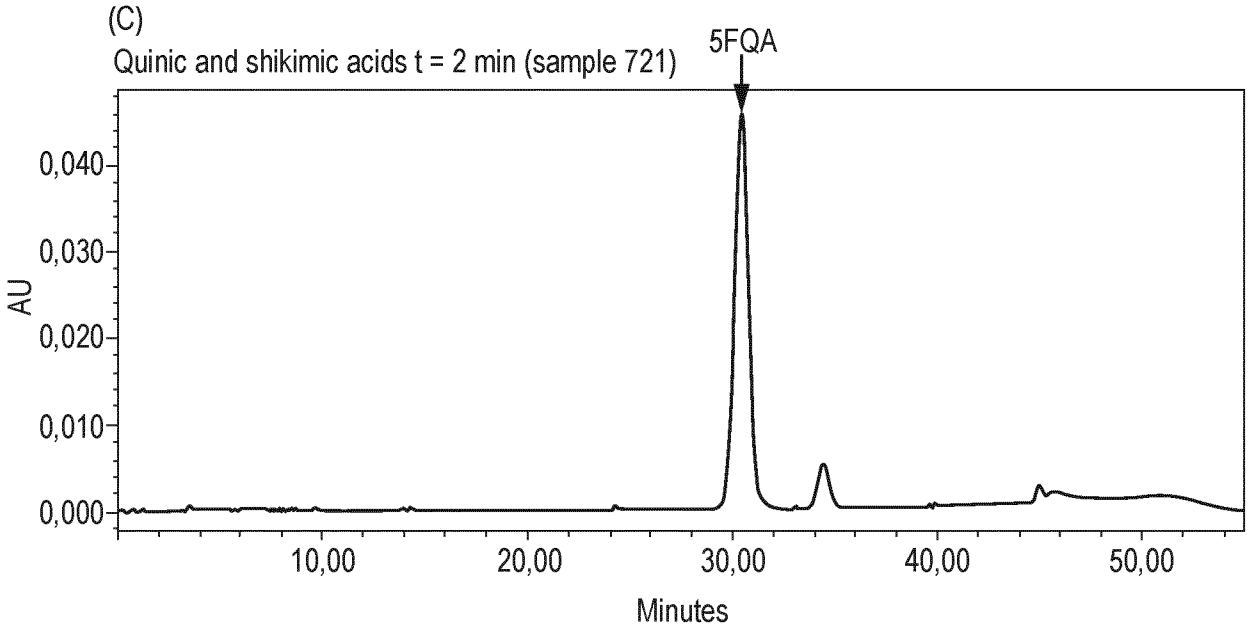

Feruloylshikimic acid
Feruloyl-CoA
326,2
253,9
344,1
0,060
0,055
0,050
0,045
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
AU
220,00 240,00 260,00 280,00 300,00 320,00 340,00 360,00 380,00
nm

FIG. 22 Cont'd


(C)
Quinic and shikimic acids t = 2 min (sample 721)
5FQA
0,040
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

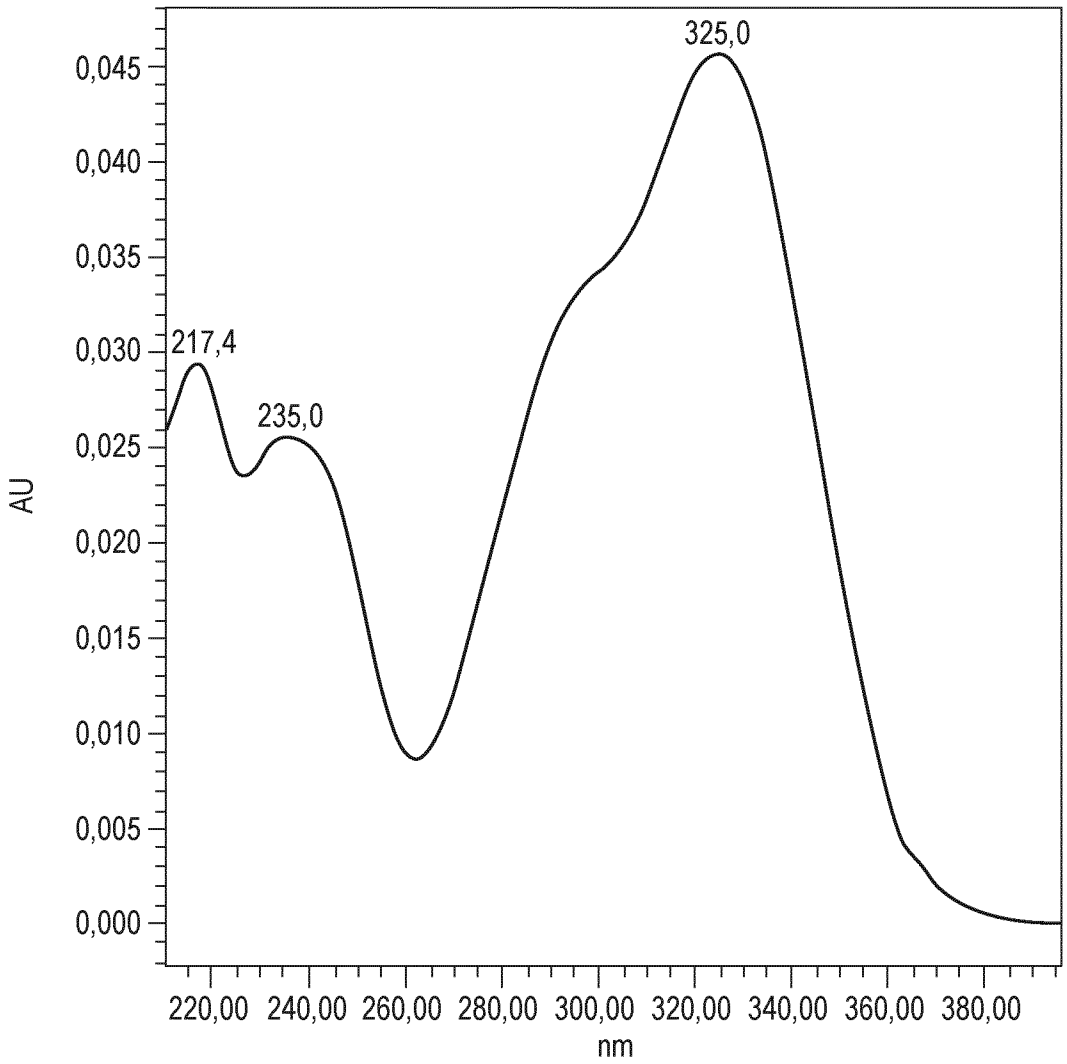

325,0
217,4
235,0
0,045
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
AU
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 22 Cont'd

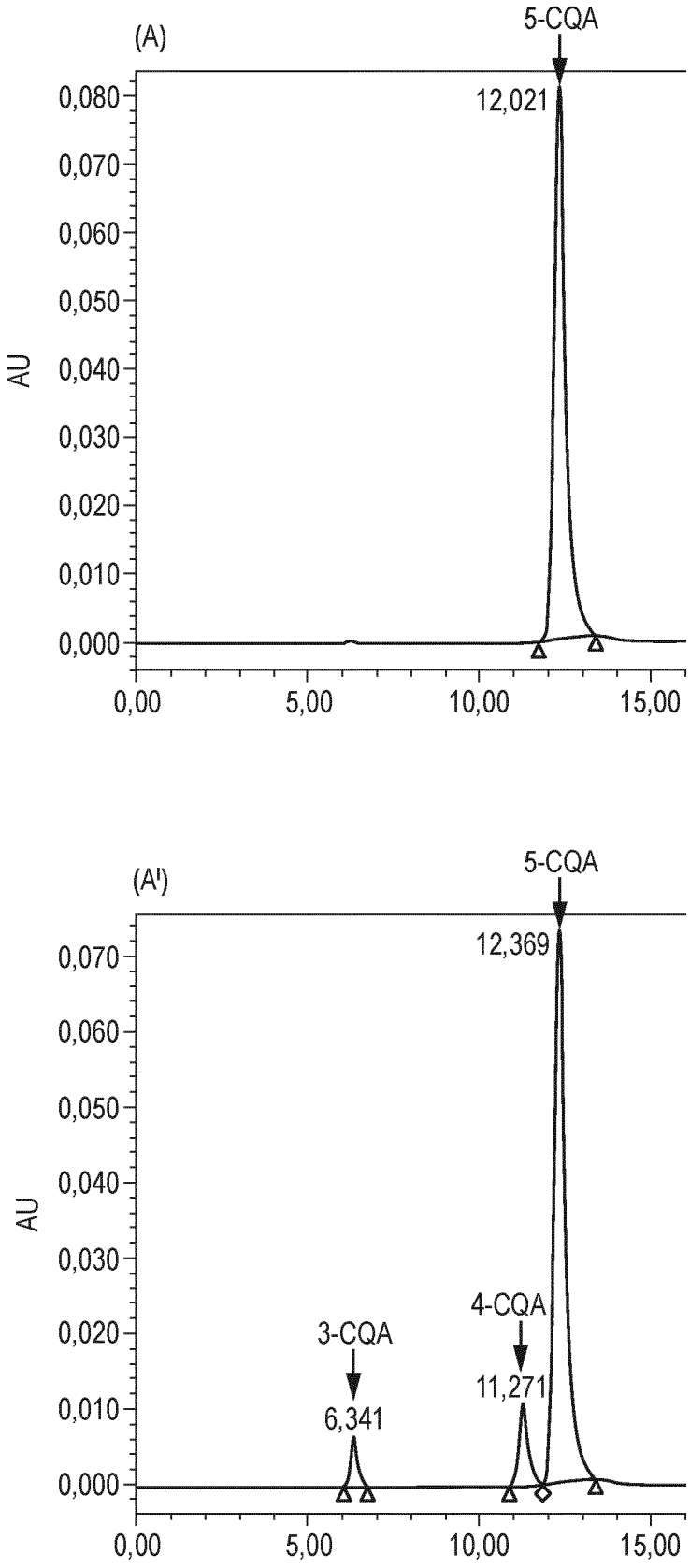

(A)
5-CQA
12,021
AU
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
0,00
5,00
10,00
15,00
(A')
5-CQA
12,369
4-CQA
11,271
3-CQA
6,341

FIG. 23

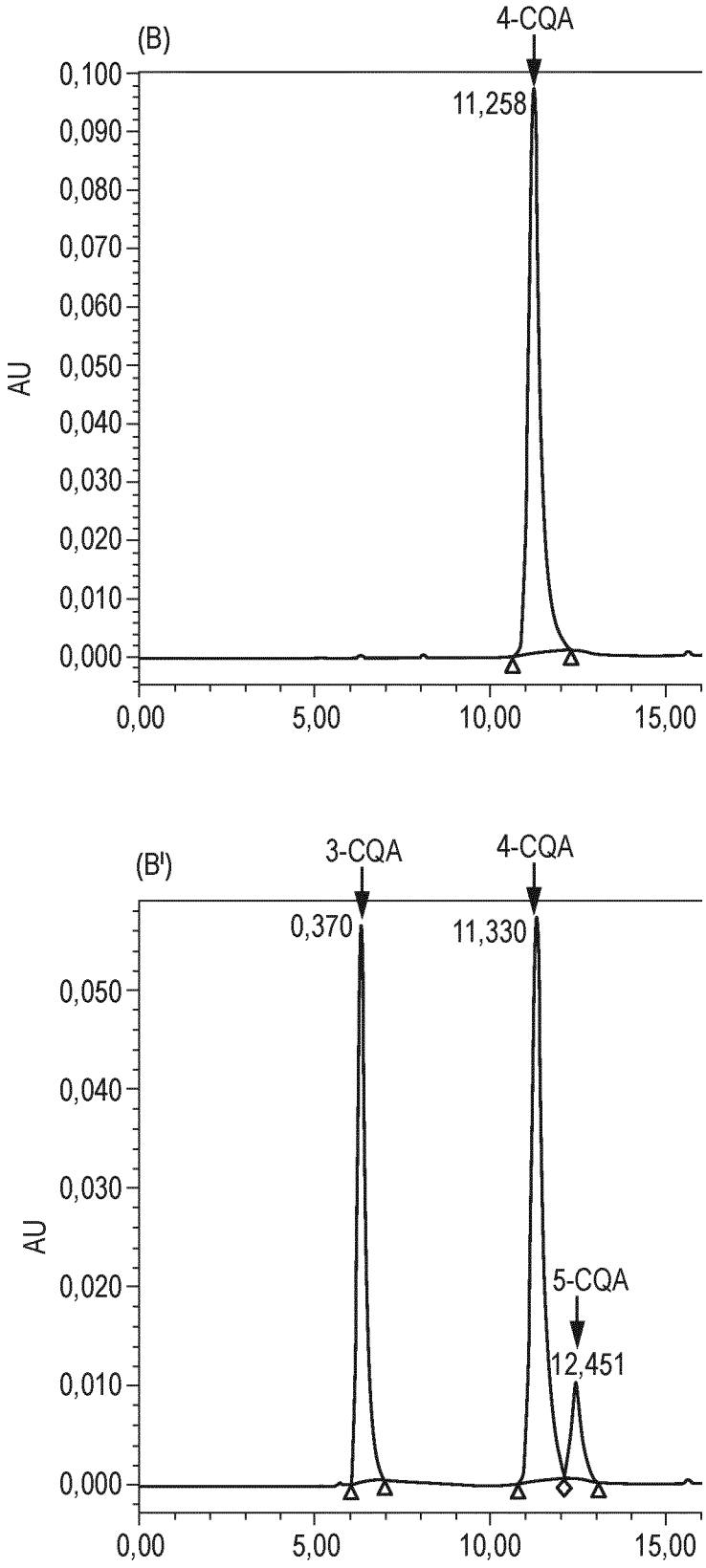

(B)
4-CQA
11,258
AU
0,100
0,090
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
0,00
5,00
10,00
15,00
(B')
3-CQA
4-CQA
0,370
11,330
5-CQA
12,451
AU
0,050
0,040
0,030
0,020
0,010
0,000
0,00
5,00
10,00
15,00

FIG. 23 Cont'd

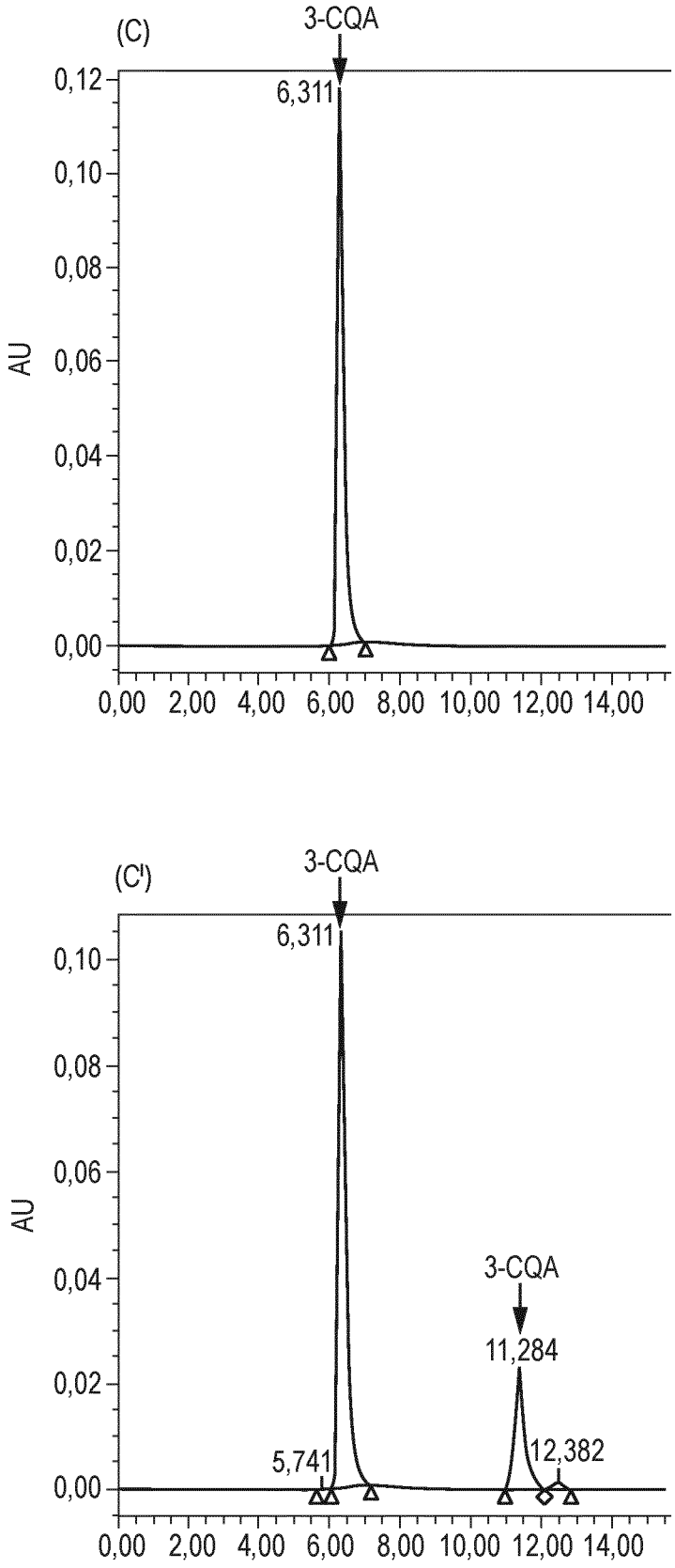

(C)
3-CQA
6,311
0,12
0,10
0,08
0,06
0,04
0,02
0,00
AU
0,00 2,00 4,00 6,00 8,00 10,00 12,00 14,00
(C')
3-CQA
6,311
0,10
0,08
0,06
0,04
0,02
0,00
AU
3-CQA
11,284
5,741
12,382
0,00 2,00 4,00 6,00 8,00 10,00 12,00 14,00

FIG. 23 Cont'd

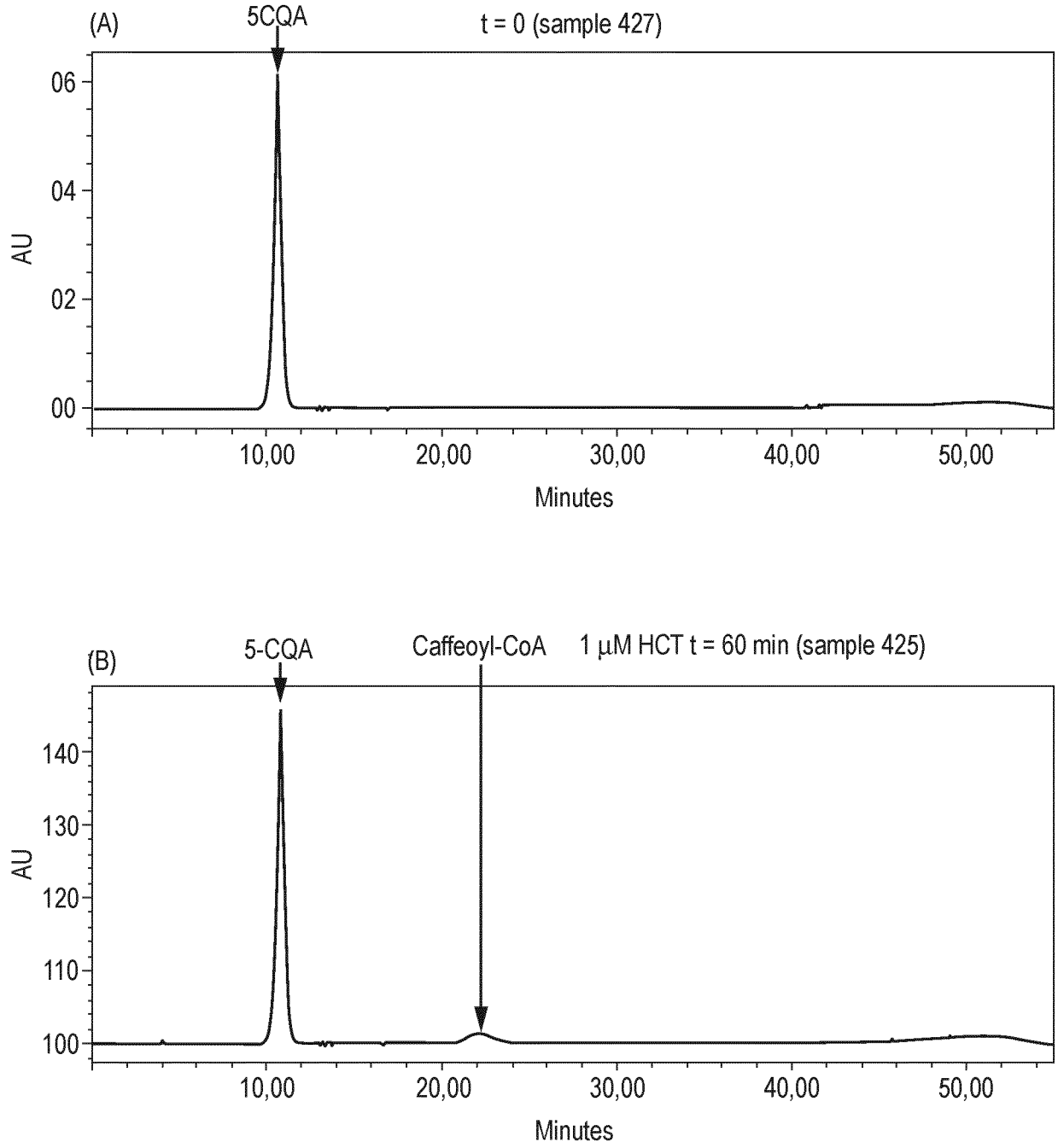
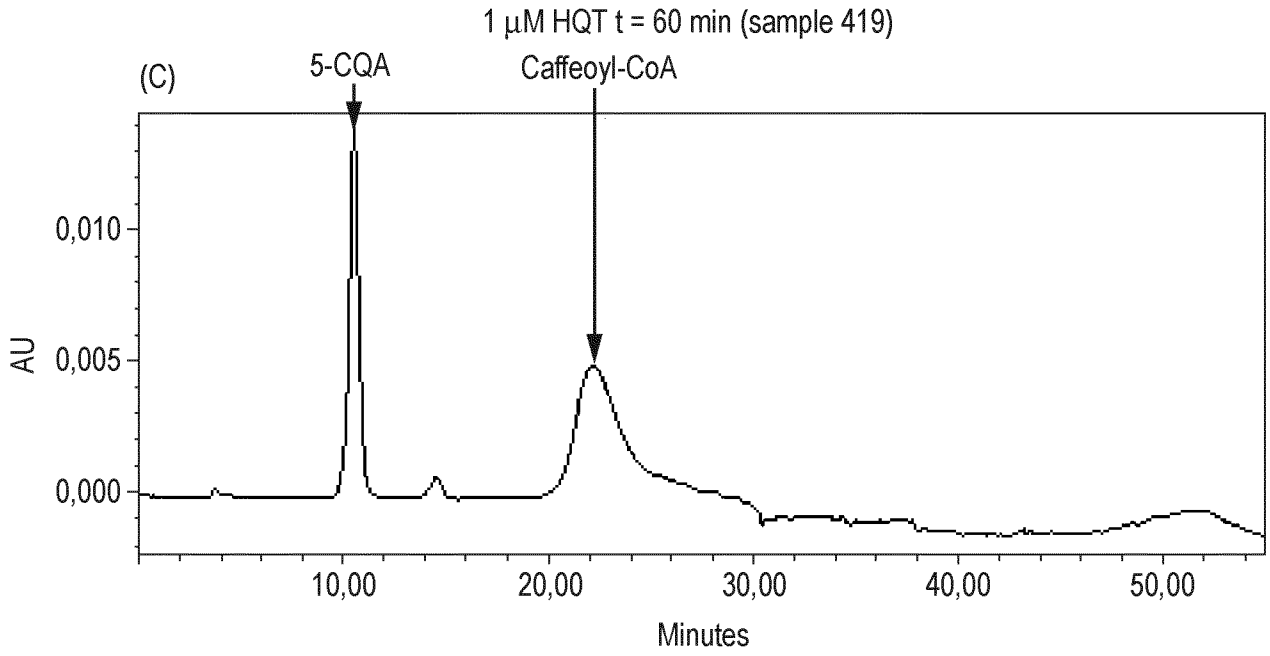

(A)
5CQA
t = 0 (sample 427)
06
04
02
00
AU
10,00
20,00
30,00
40,00
50,00
Minutes
(B)
5-CQA
Caffeoyl-CoA
1 µM HCT t = 60 min (sample 425)
140
130
120
110
100
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 24


1 µM HQT t = 60 min (sample 419)
(C)
5-CQA
Caffeoyl-CoA
0,010
0,005
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

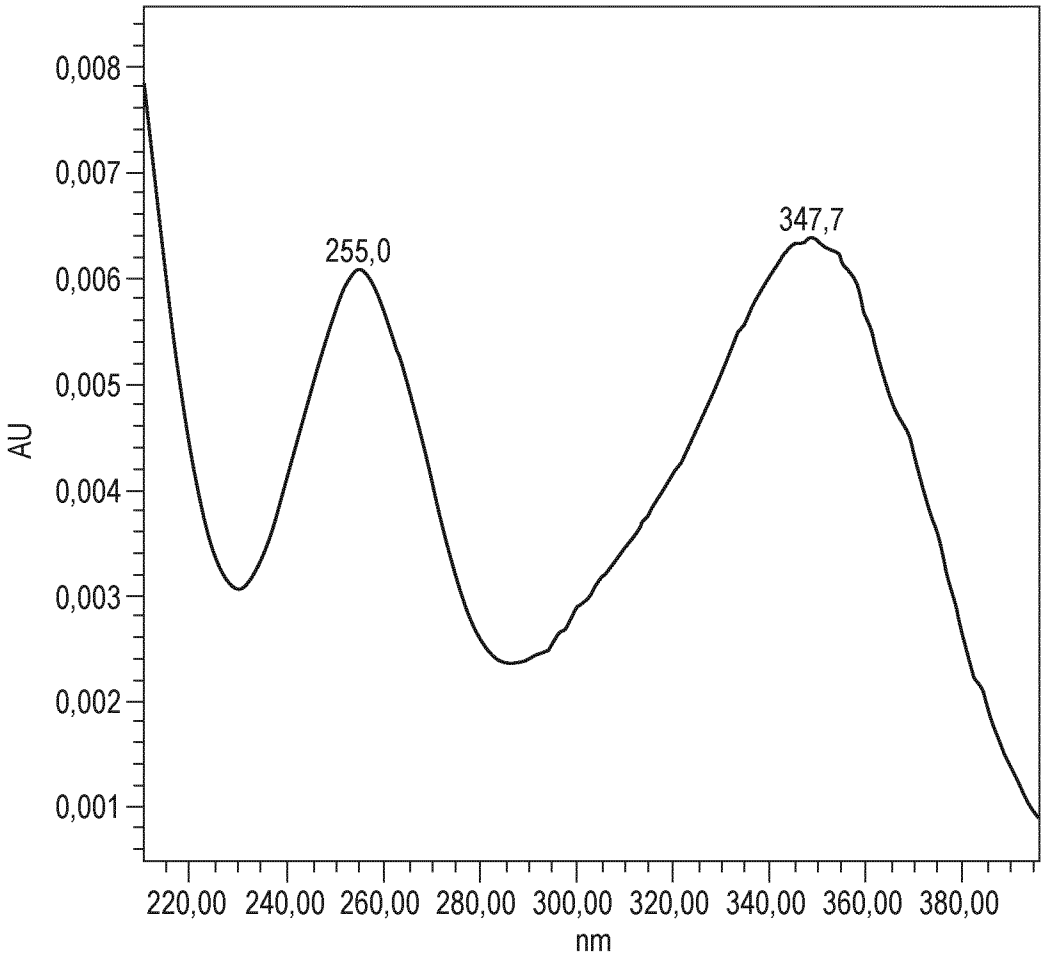

0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
AU
255,0
347,7
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 24 Cont'd

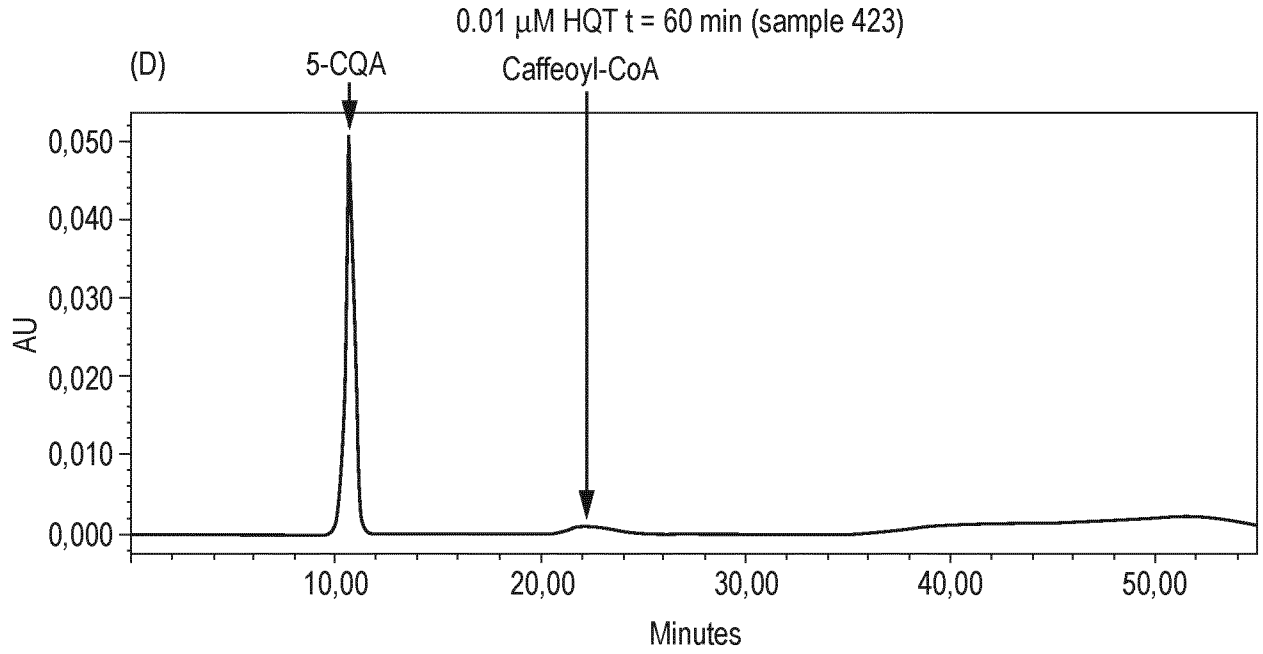

0.01 µM HQT t = 60 min (sample 423)
(D)
5-CQA
Caffeoyl-CoA
0,050
0,040
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 24 Cont'd

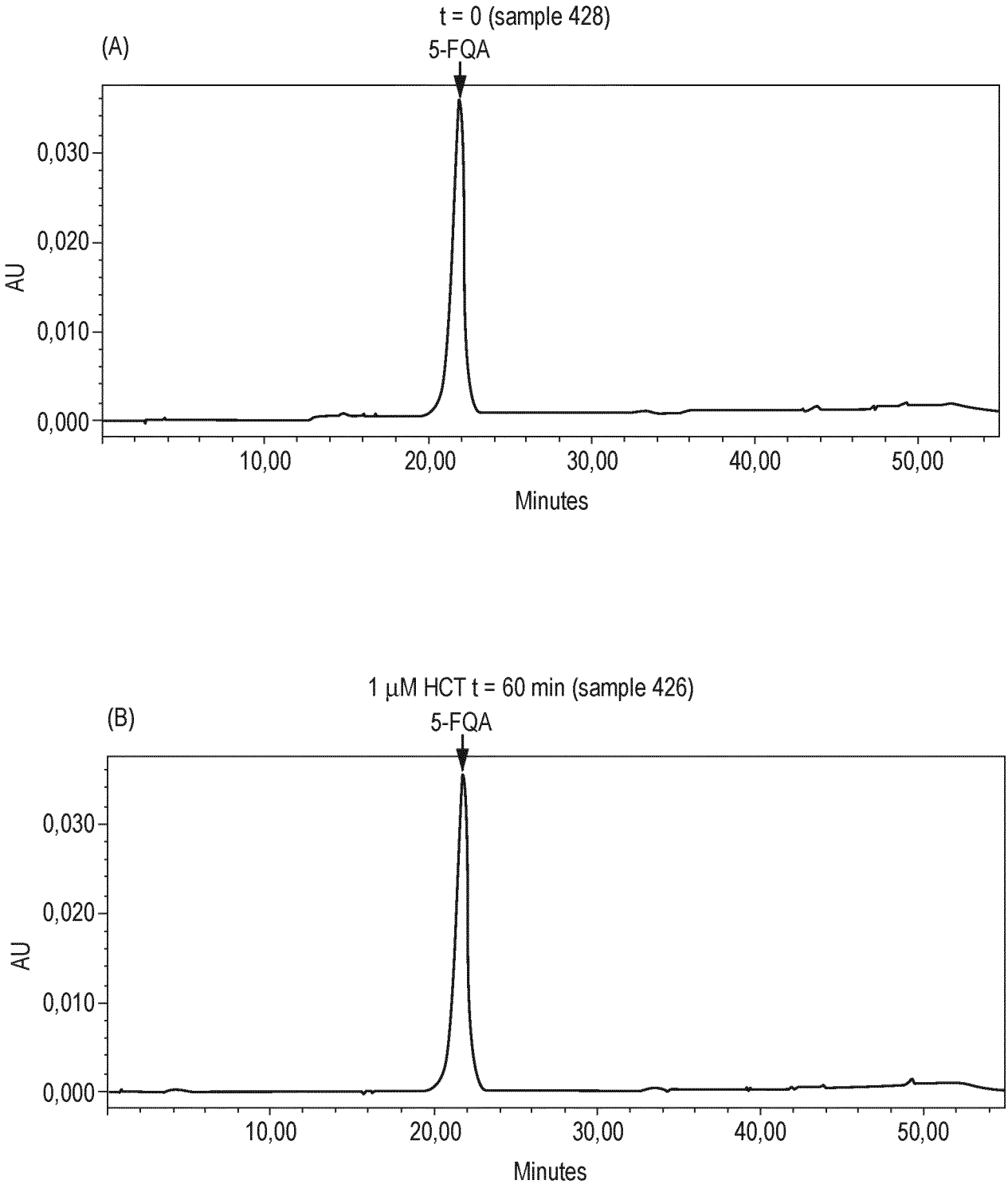
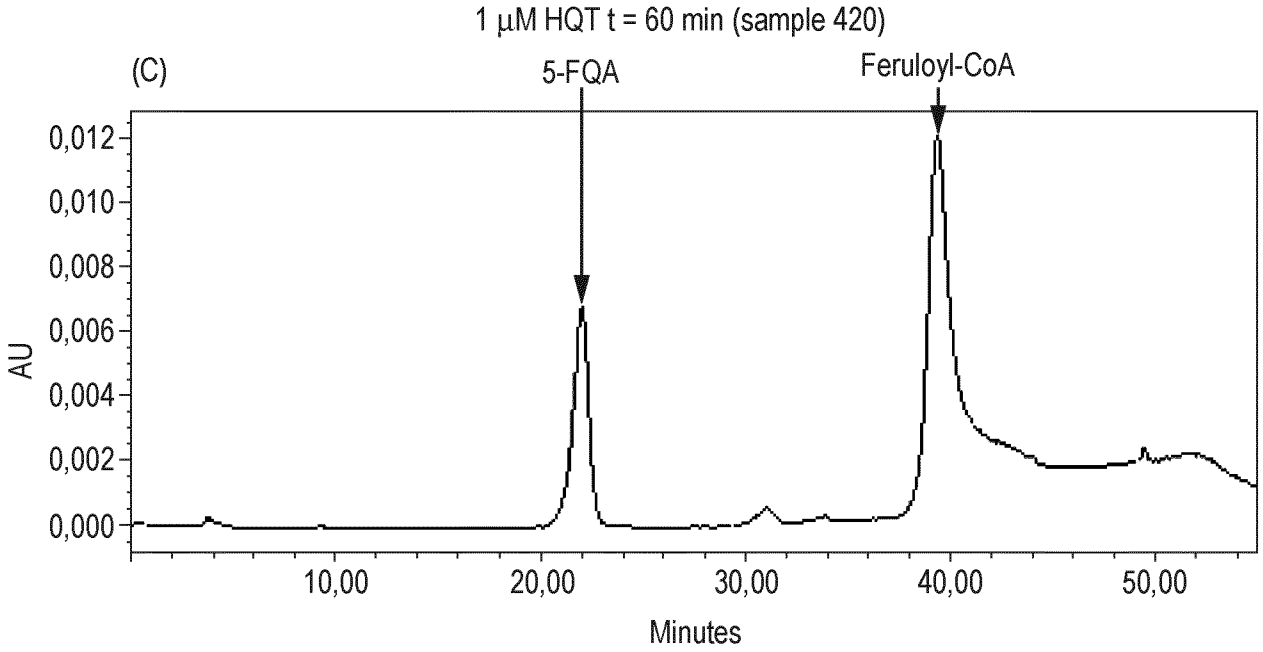

t = 0 (sample 428)
(A)
5-FQA
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes
1 µM HCT t = 60 min (sample 426)
(B)
5-FQA
0,030
0,020
0,010
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 25


1 µM HQT t = 60 min (sample 420)
(C)
5-FQA
Feruloyl-CoA
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
10,00
20,00
30,00
40,00
50,00
Minutes

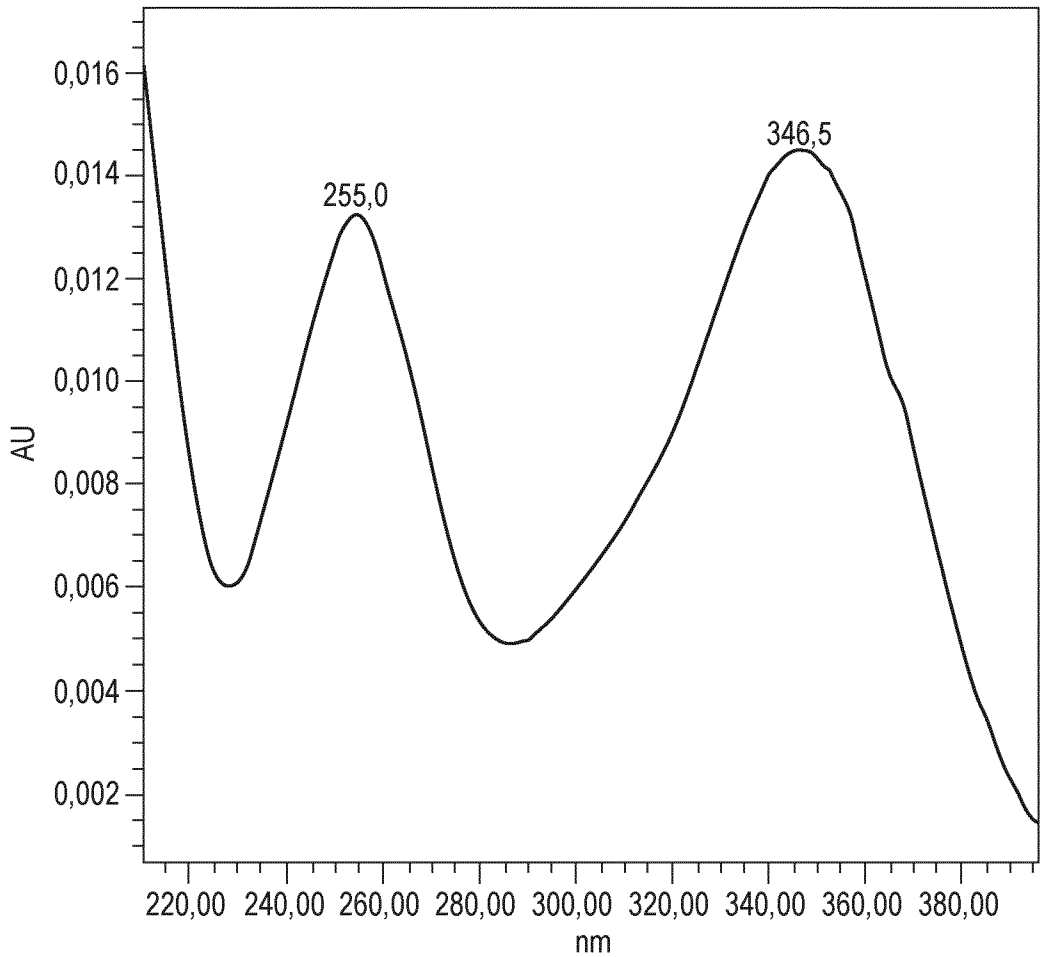

0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
AU
255,0
346,5
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 25 Cont'd

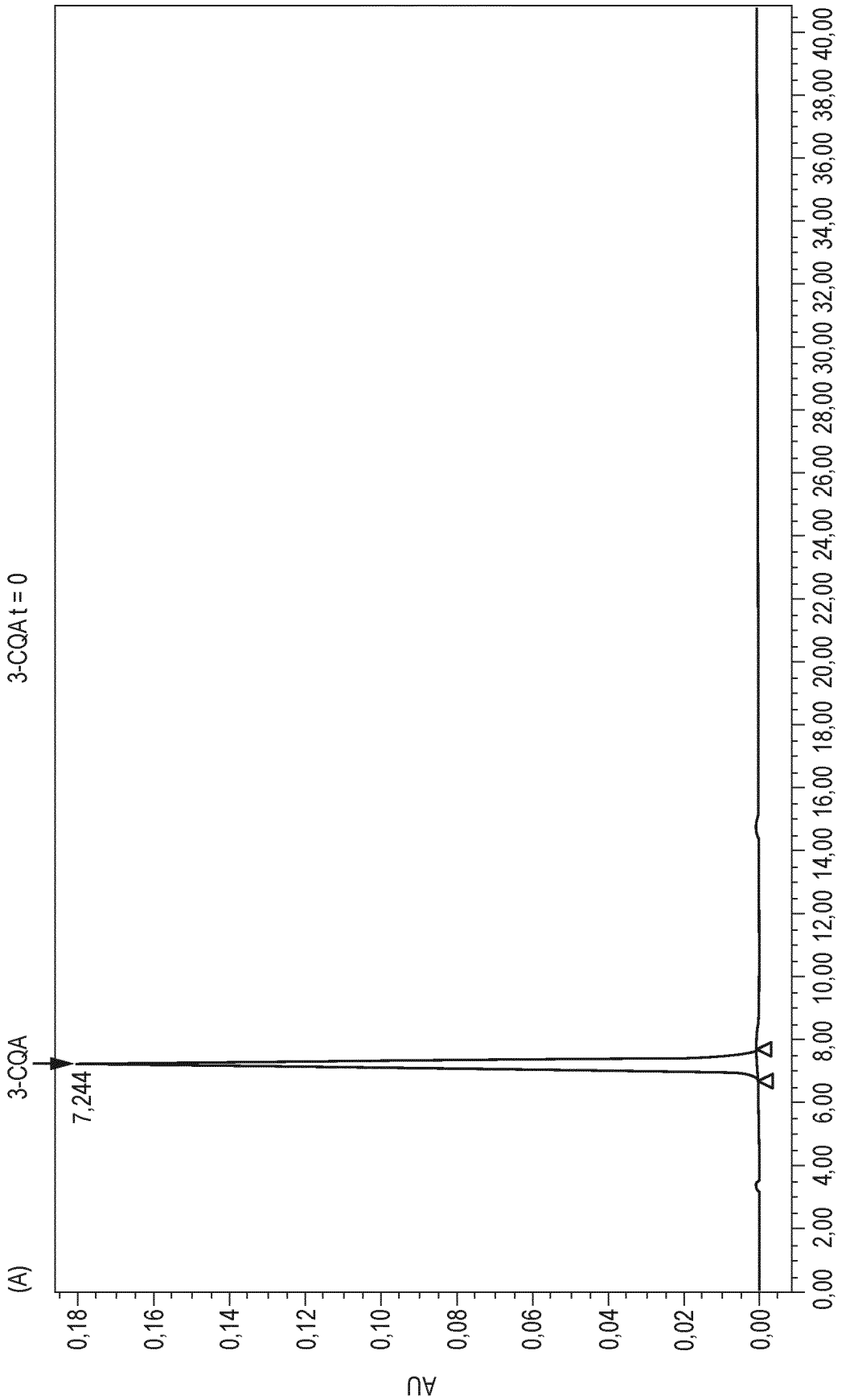

(A)
3-CQA
7,244
3-CQA t = 0
AU
0,18
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00 2,00 4,00 6,00 8,00 10,00 12,00 14,00 16,00 18,00 20,00 22,00 24,00 26,00 28,00 30,00 32,00 34,00 36,00 38,00 40,00
Minutes

FIG. 26

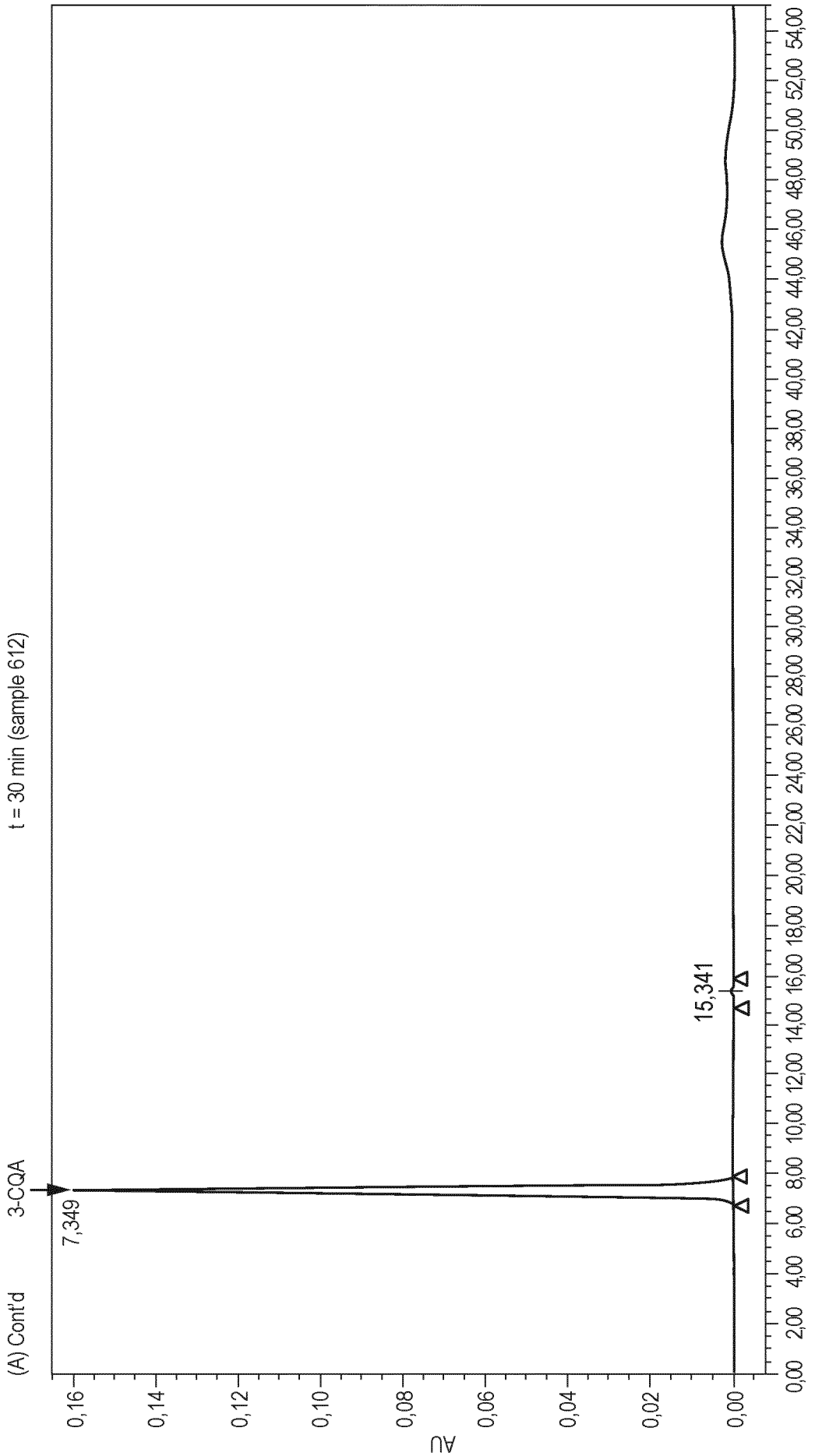

(A) Cont'd
3-CQA
t = 30 min (sample 612)
7,349
15,341
AU
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0,00 2,00 4,00 6,00 8,00 10,00 12,00 14,00 16,00 18,00 20,00 22,00 24,00 26,00 28,00 30,00 32,00 34,00 36,00 38,00 40,00 42,00 44,00 46,00 48,00 50,00 52,00 54,00
Minutes

FIG. 26 Cont'd

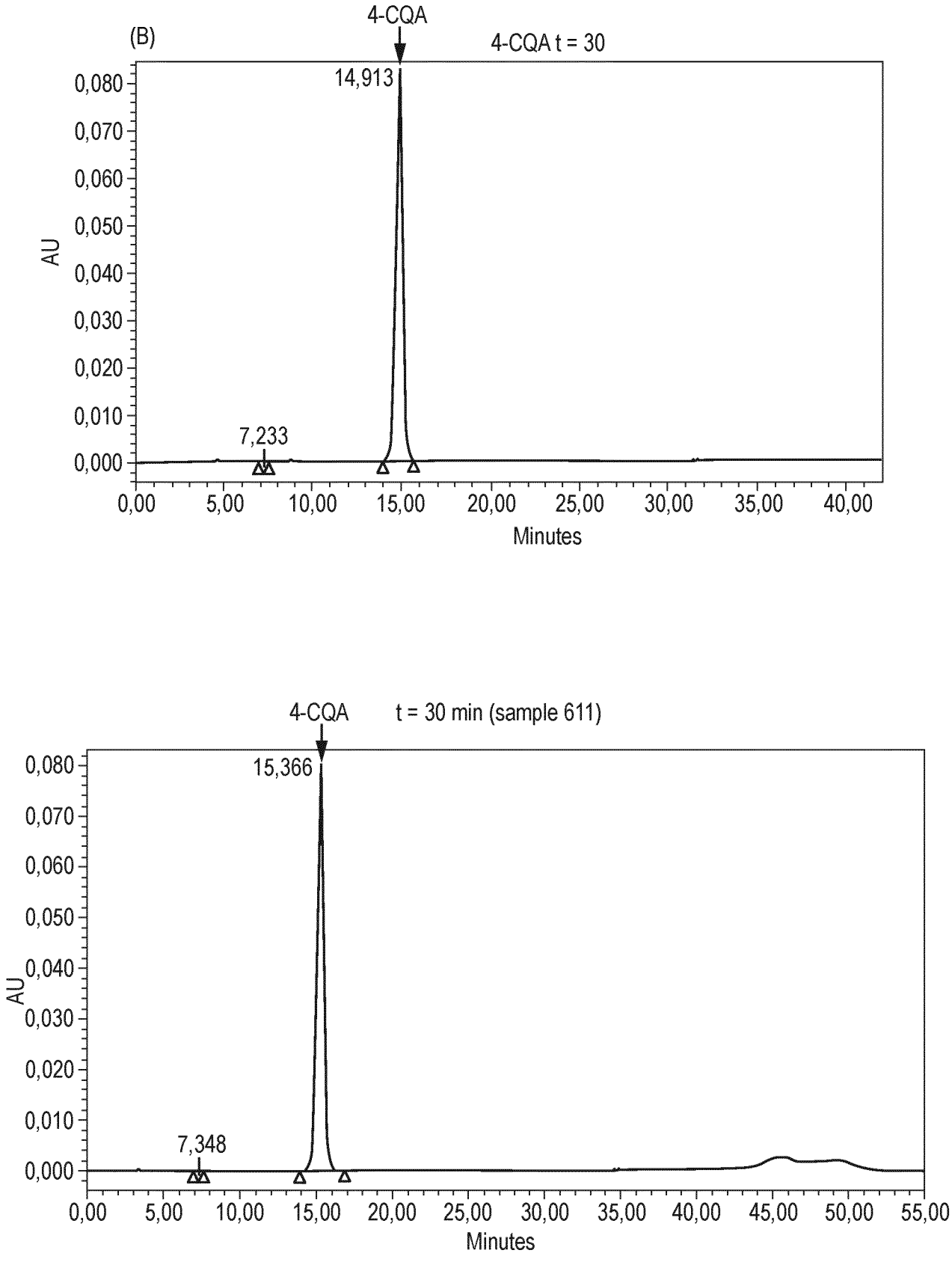

(B)
4-CQA
4-CQA t = 30
14,913
7,233
AU
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
Minutes
4-CQA
t = 30 min (sample 611)
15,366
7,348
AU
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 26 Cont'd

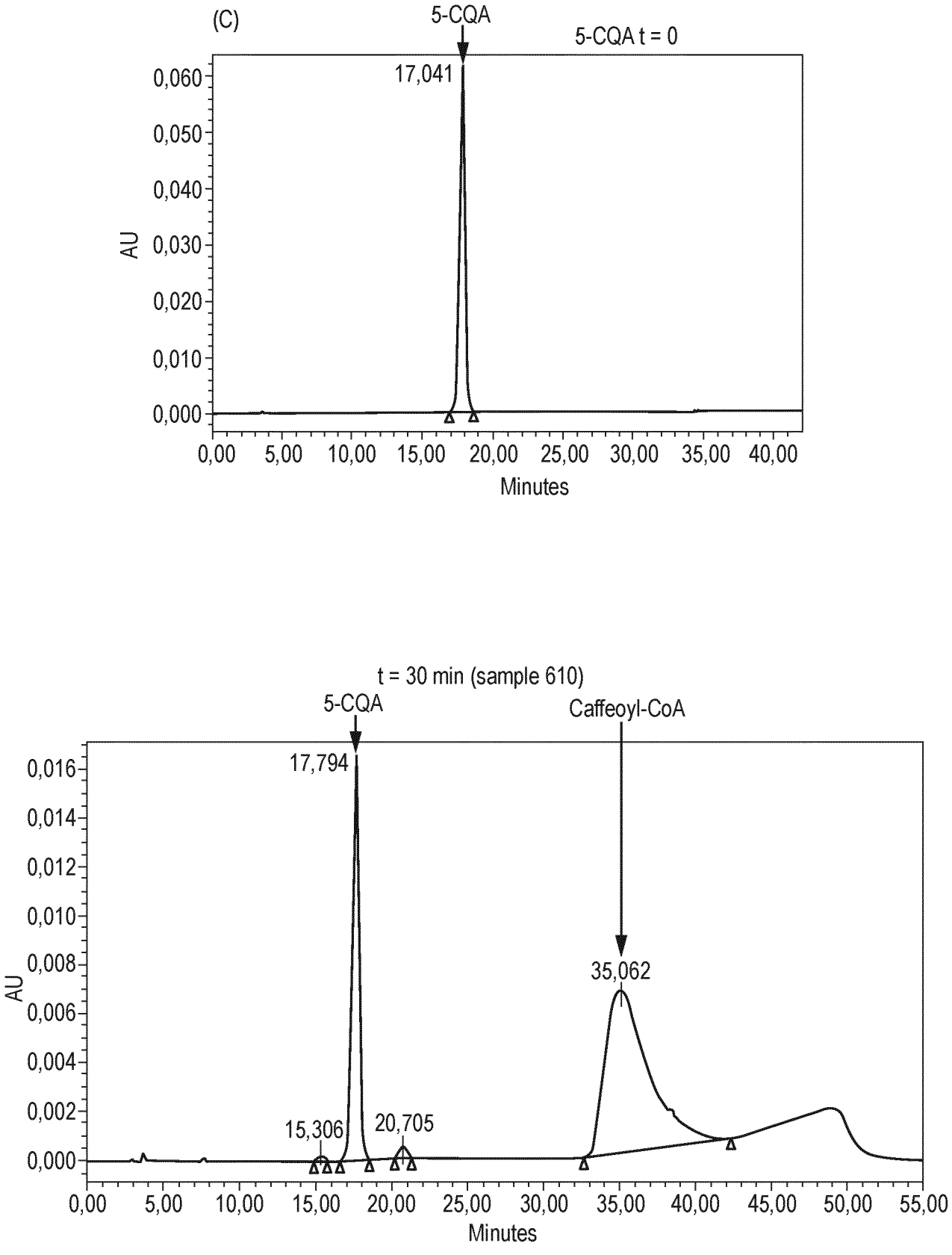

(C)
5-CQA
5-CQA t = 0
17,041
AU
0,060
0,050
0,040
0,030
0,020
0,010
0,000
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00
Minutes
t = 30 min (sample 610)
5-CQA
Caffeoyl-CoA
17,794
35,062
15,306
20,705
AU
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,00 5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 26 Cont'd

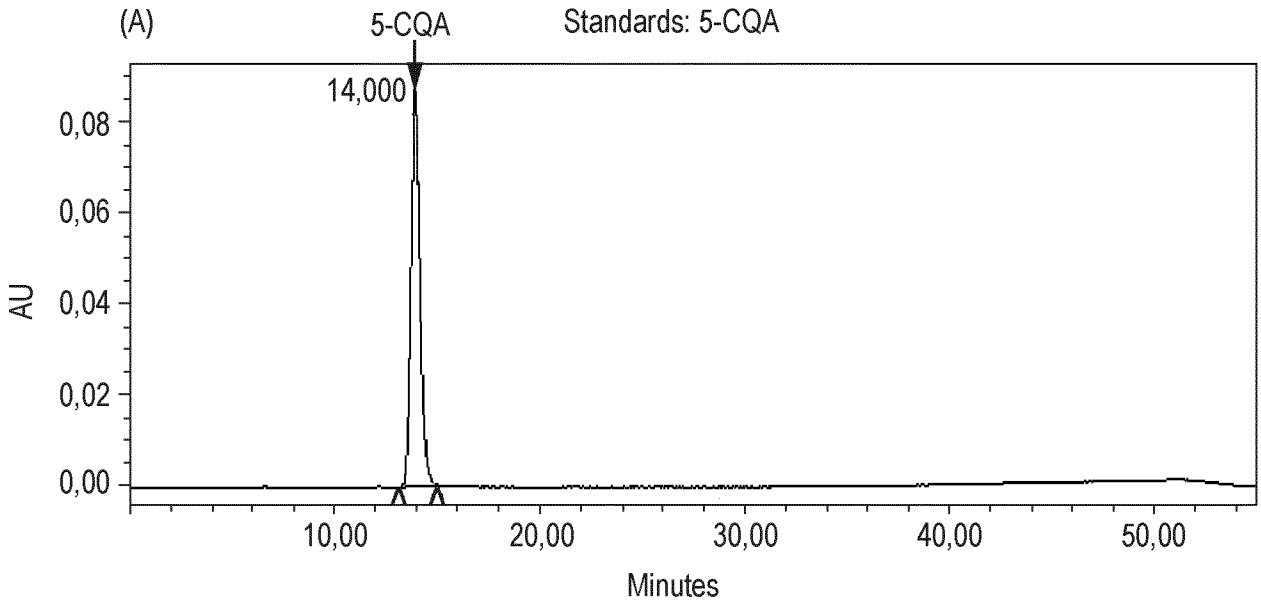

(A)
5-CQA
Standards: 5-CQA
14,000
0,08
0,06
0,04
0,02
0,00
AU
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 27

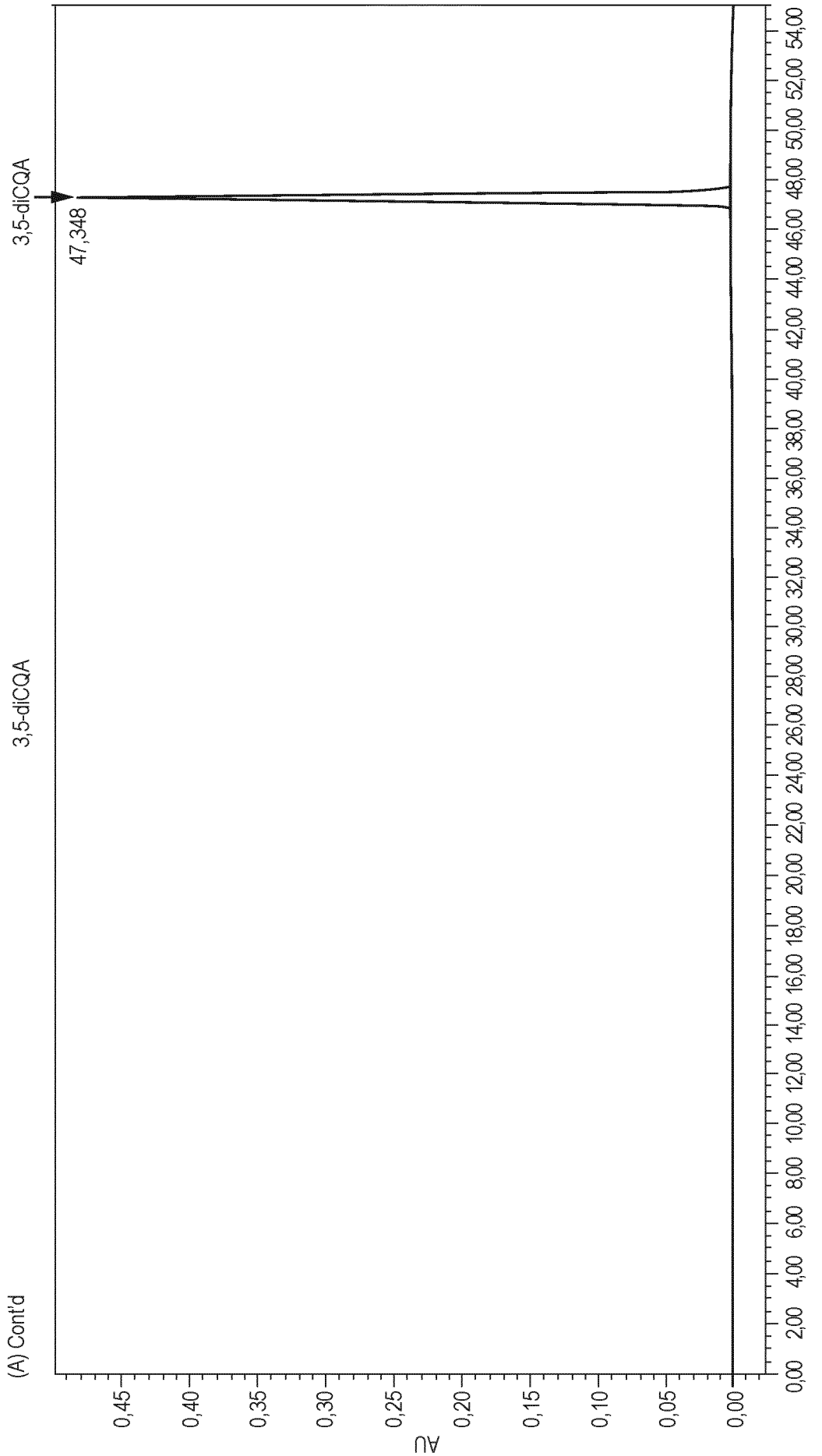

(A) Cont'd
3,5-diCQA
3,5-diCQA
47,348
AU
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0,00 2,00 4,00 6,00 8,00 10,00 12,00 14,00 16,00 18,00 20,00 22,00 24,00 26,00 28,00 30,00 32,00 34,00 36,00 38,00 40,00 42,00 44,00 46,00 48,00 50,00 52,00 54,00
Minutes

FIG. 27 Cont'd

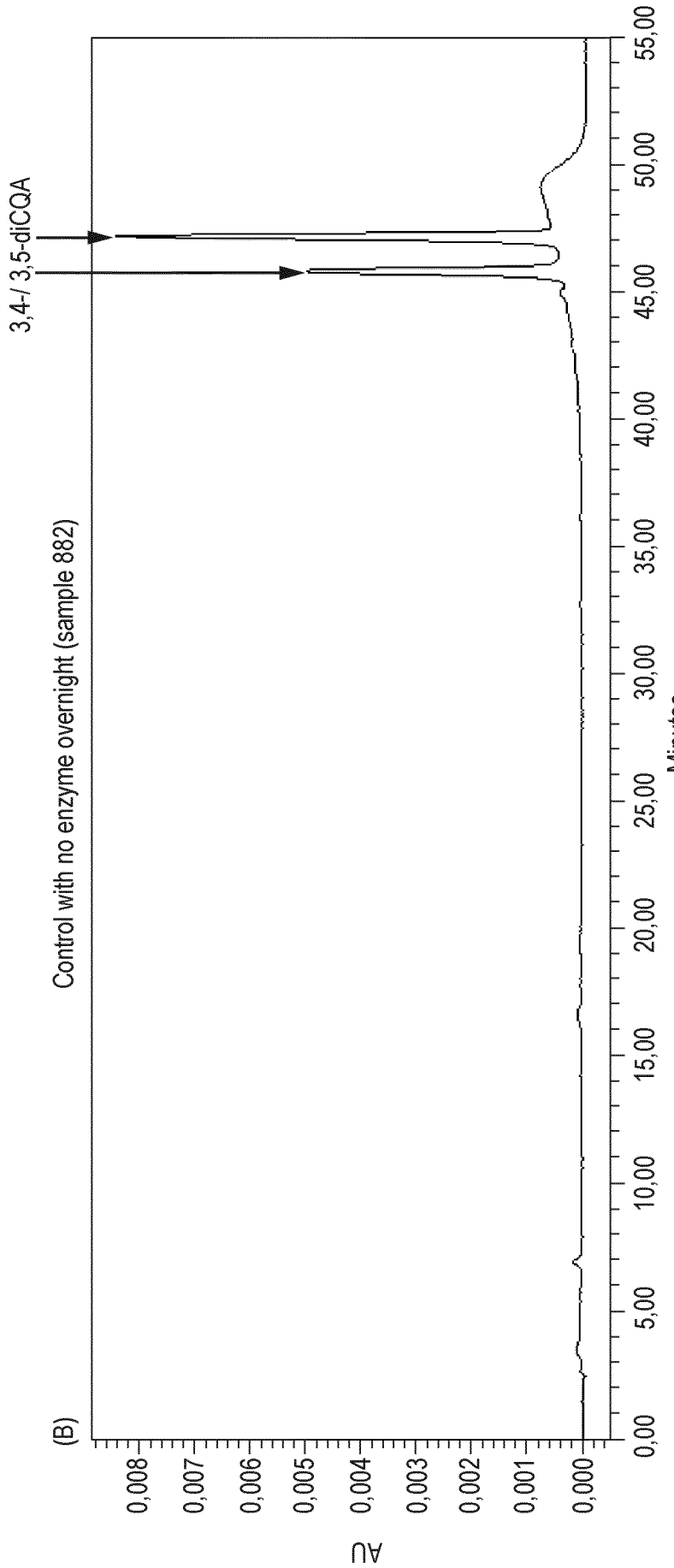
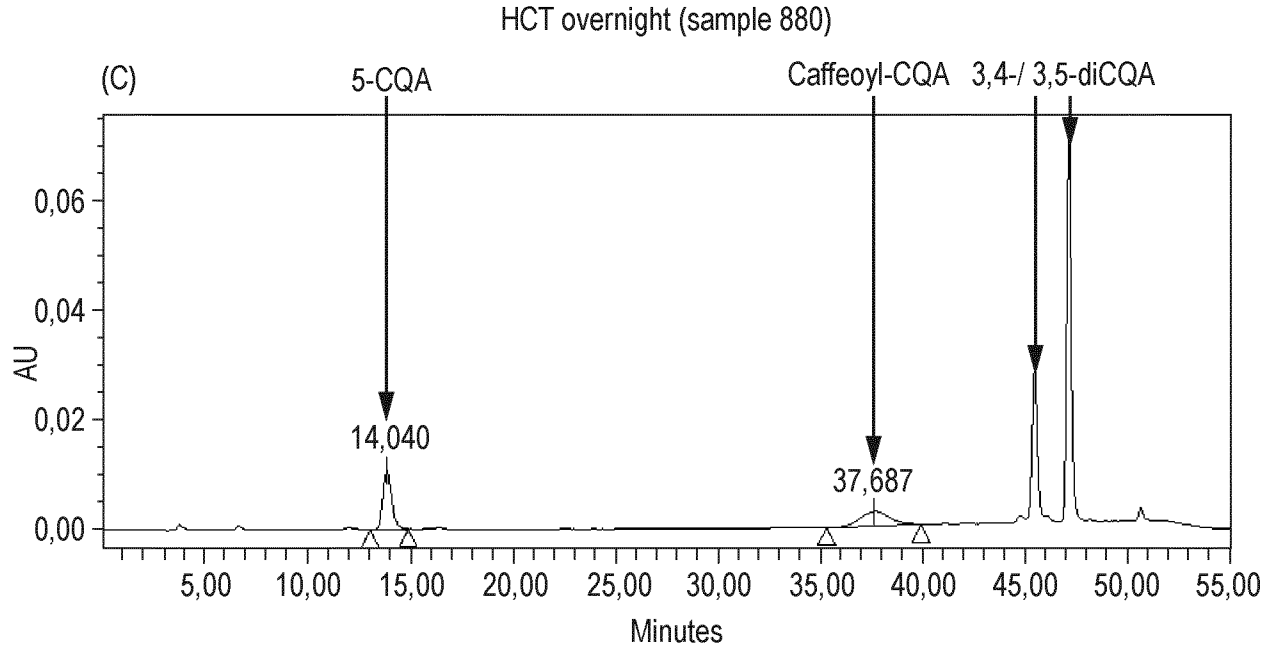

(B)
Control with no enzyme overnight (sample 882)
3,4-/ 3,5-diCQA
AU
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 27 Cont'd


HCT overnight (sample 880)
(C)
5-CQA
Caffeoyl-CQA
3,4-/ 3,5-diCQA
0,06
0,04
0,02
0,00
AU
14,040
37,687
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

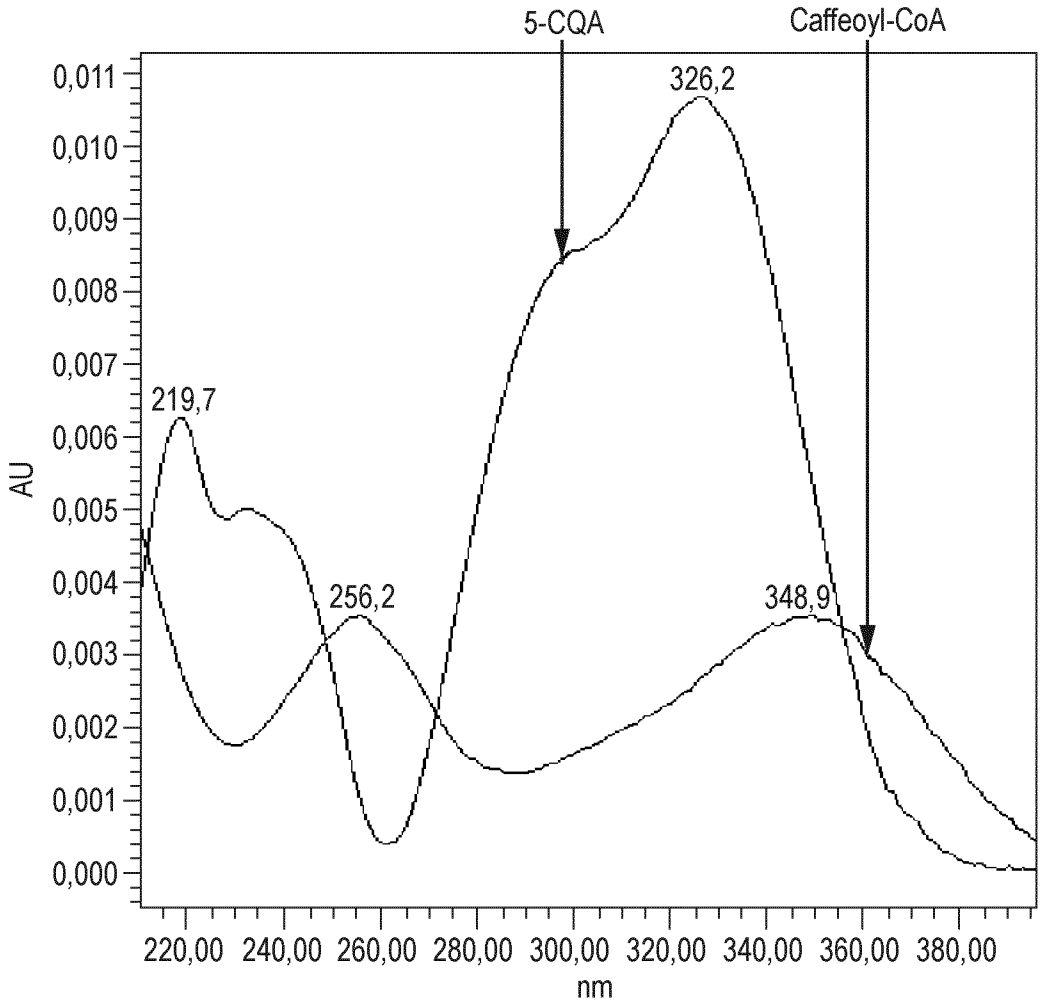

5-CQA
Caffeoyl-CoA
326,2
0,011
0,010
0,009
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
AU
219,7
256,2
348,9
220,00
240,00
260,00
280,00
300,00
320,00
340,00
360,00
380,00
nm

FIG. 27 Cont'd

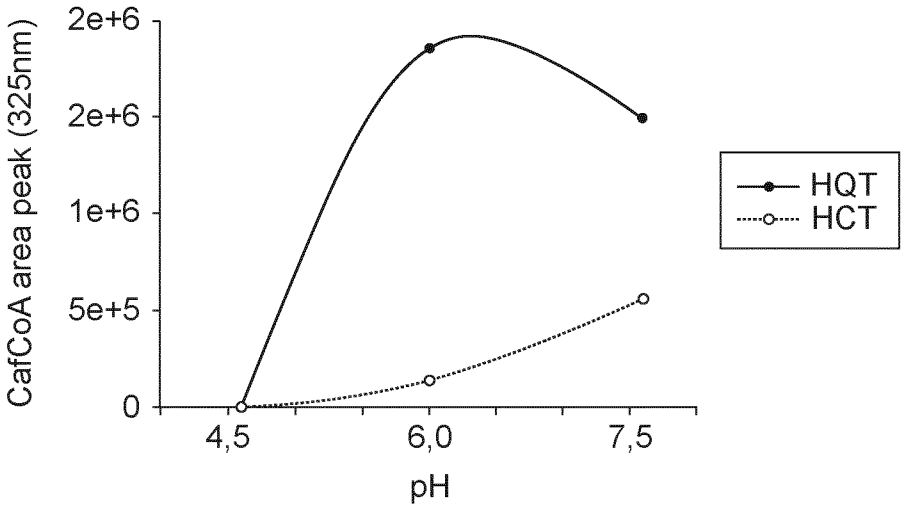

CafCoA area peak (325nm)
2e+6
2e+6
1e+6
5e+5
0
4,5
6,0
7,5
pH
HQT
HCT

FIG. 28

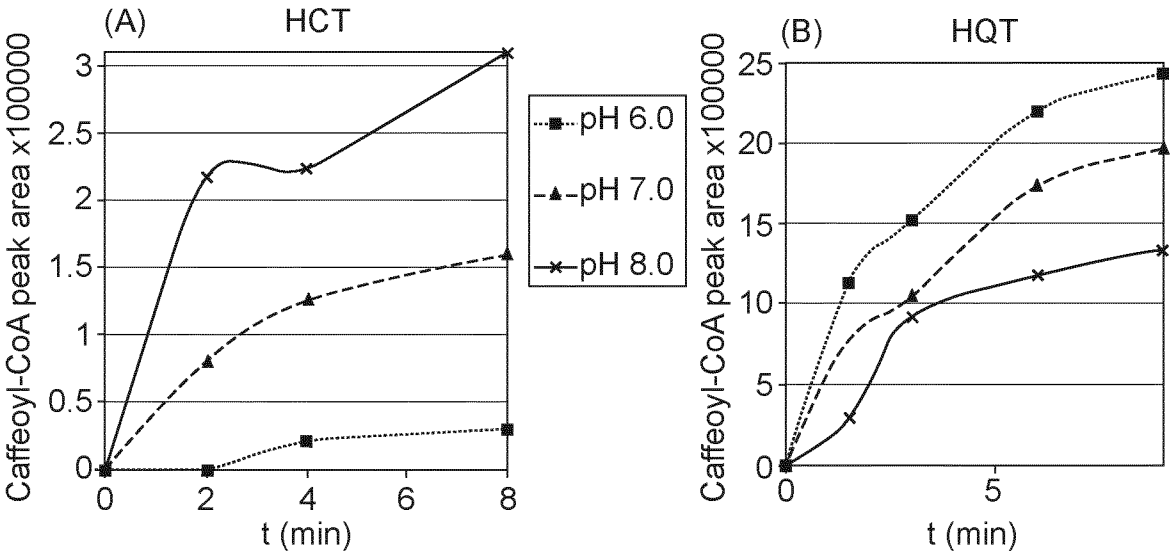

(A)
HCT
Caffeoyl-CoA peak area x100000
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
t (min)
pH 6.0
pH 7.0
pH 8.0
(B)
HQT
Caffeoyl-CoA peak area x100000
25
20
15
10
5
0
0
5
t (min)

FIG. 29

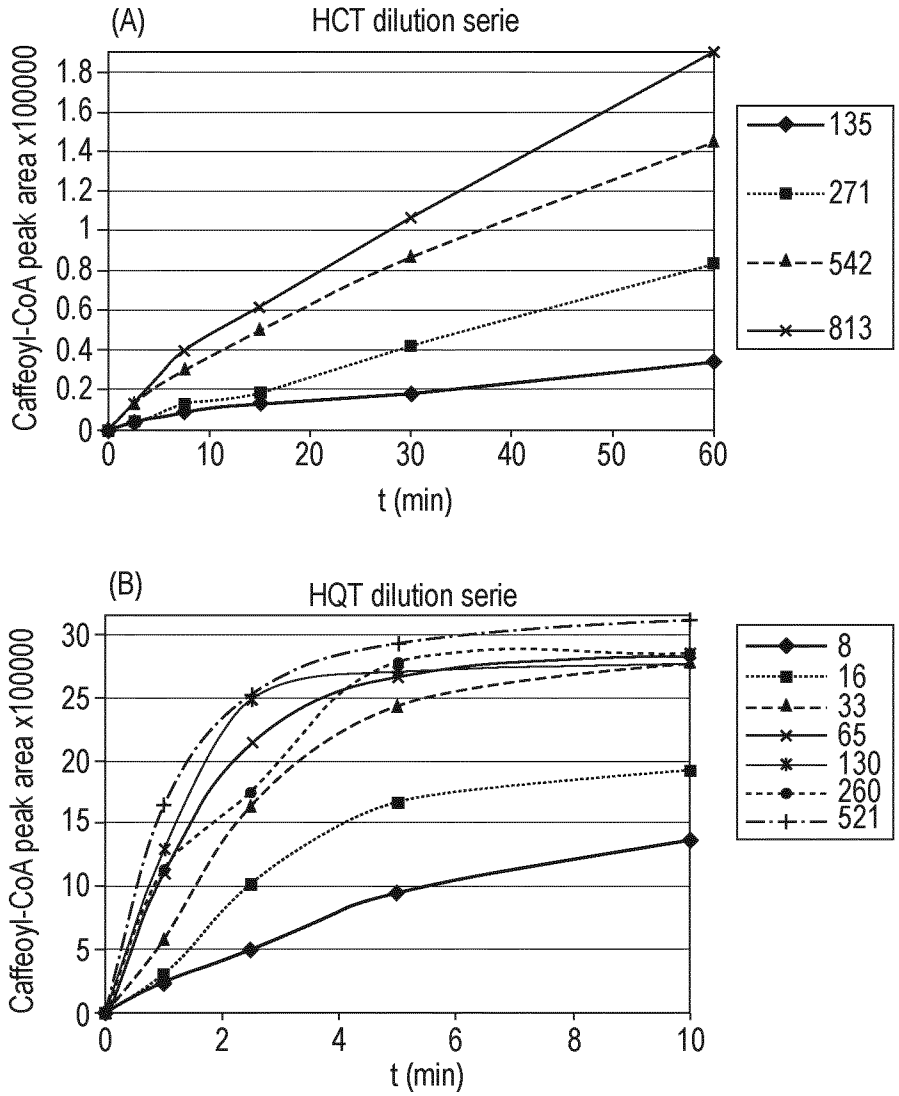

(A)
HCT dilution serie
Caffeoyl-CoA peak area x100000
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
t (min)
135
271
542
813
(B)
HQT dilution serie
Caffeoyl-CoA peak area x100000
30
25
20
15
10
5
0
0
2
4
6
8
10
t (min)
8
16
33
65
130
260
521

FIG. 30

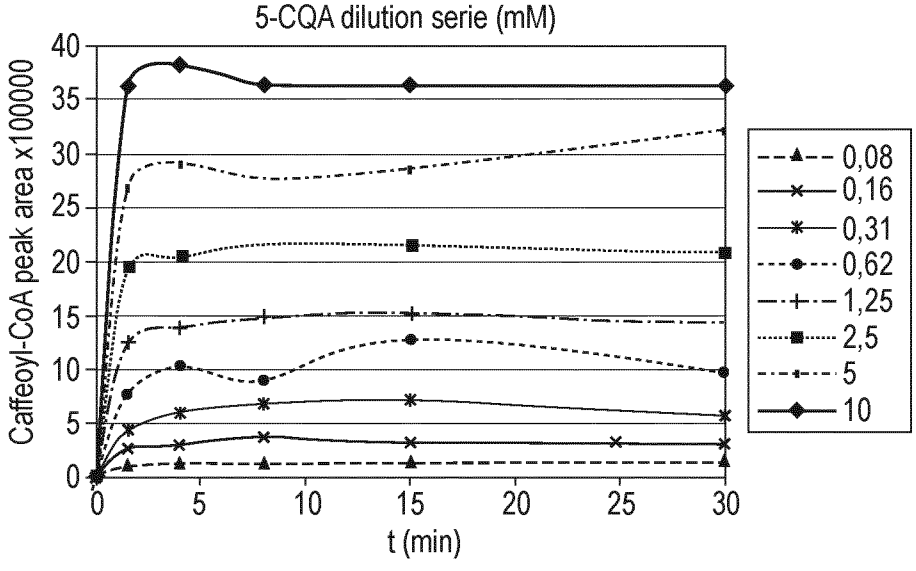

5-CQA dilution serie (mM)
Caffeoyl-CoA peak area x100000
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
t (min)
0,08
0,16
0,31
0,62
1,25
2,5
5
10

FIG. 31

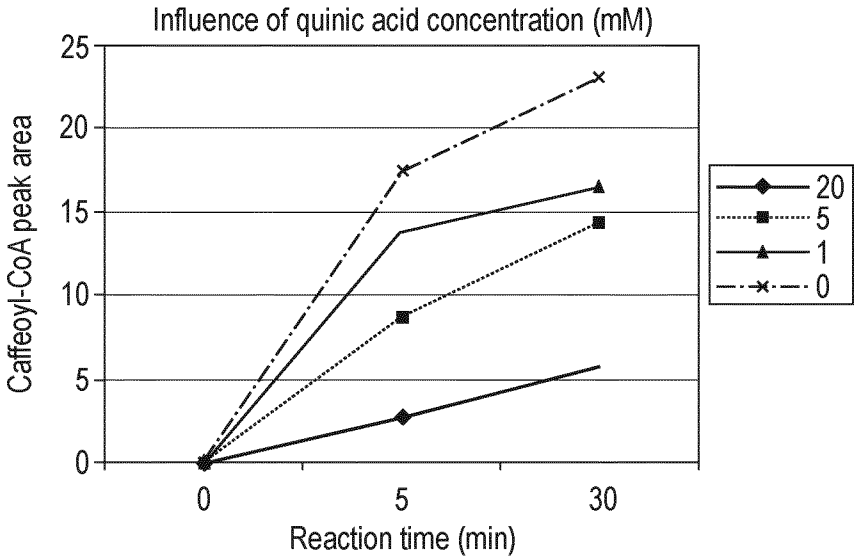
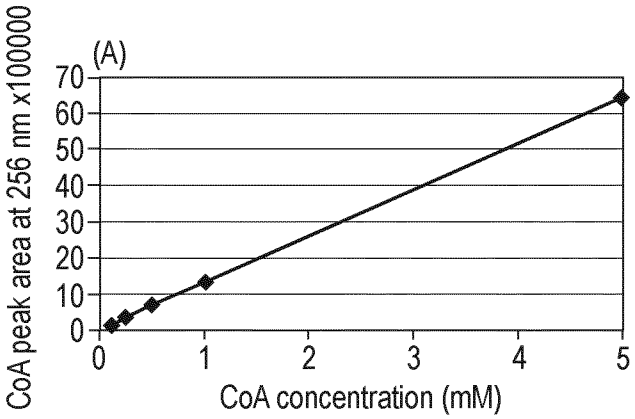

Influence of quinic acid concentration (mM)
Caffeoyl-CoA peak area
25
20
15
10
5
0
0
5
30
Reaction time (min)
20
5
1
0

FIG. 32


(A)
CoA peak area at 256 nm x100000
70
60
50
40
30
20
10
0
0
1
2
3
4
5
CoA concentration (mM)

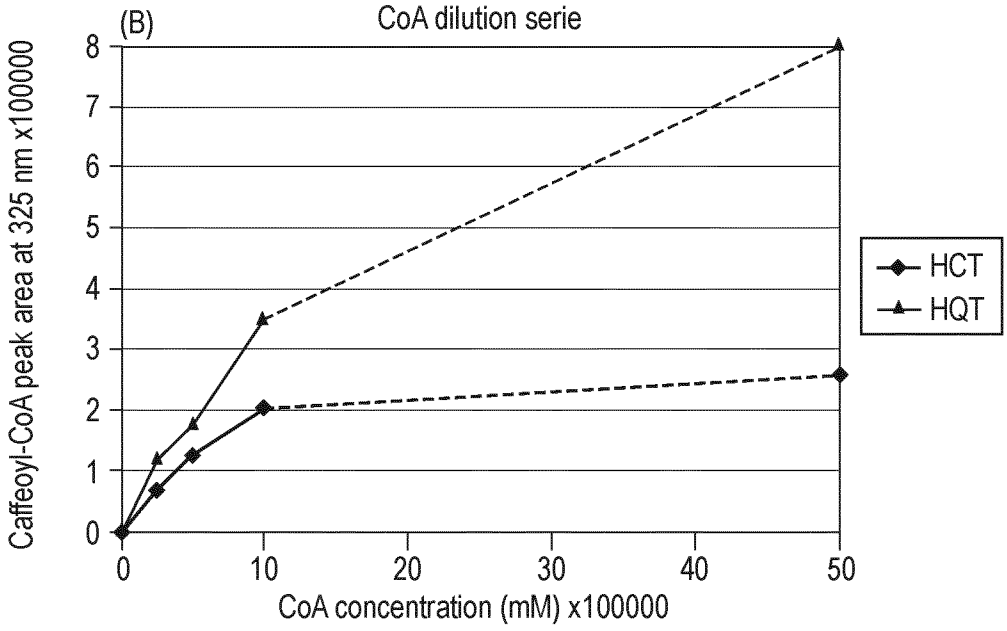

(B)
CoA dilution serie
Caffeoyl-CoA peak area at 325 nm x100000
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
CoA concentration (mM) x100000
HCT
HQT

FIG. 33

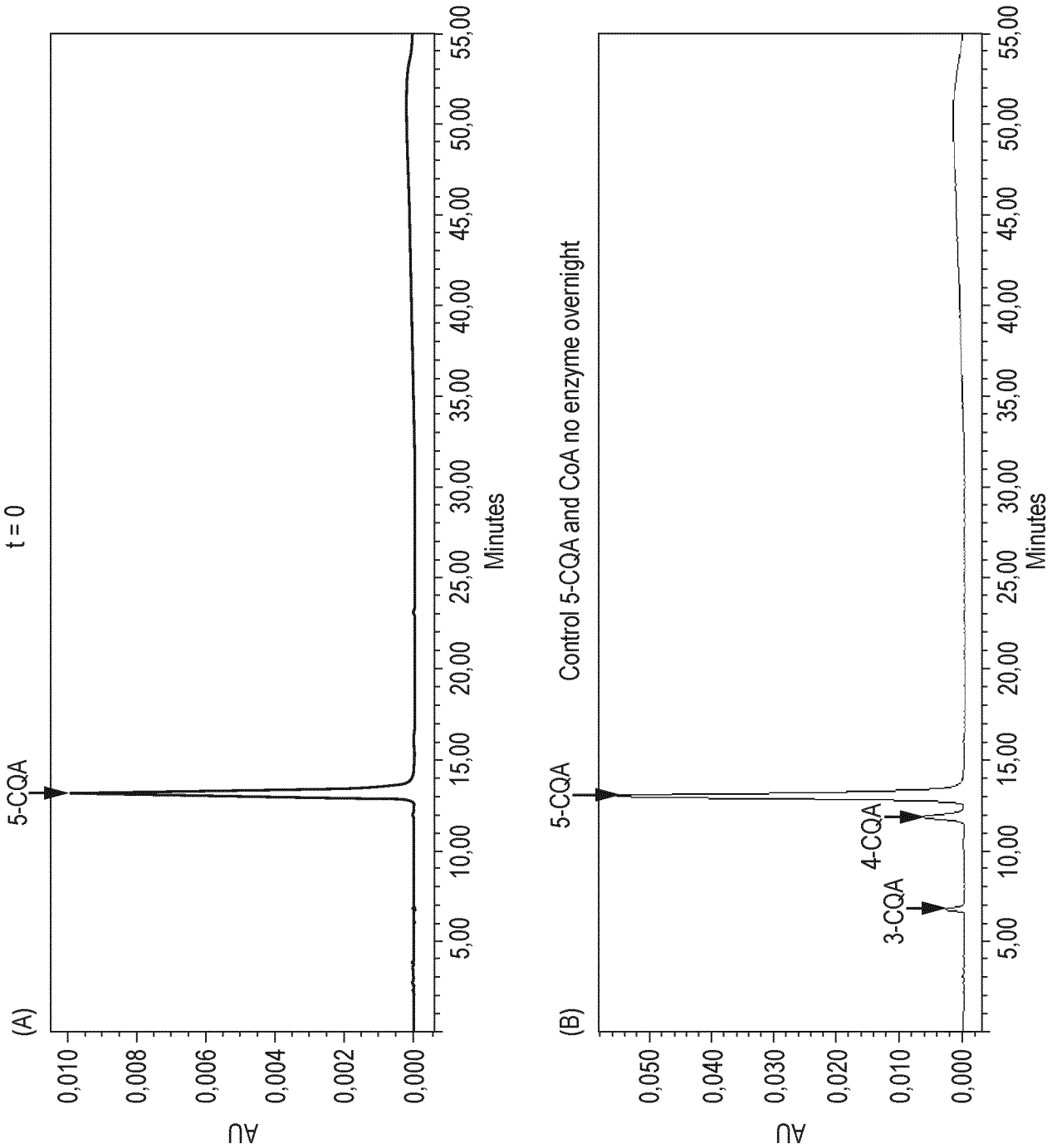

(A)
t = 0
5-CQA
AU
0,010
0,008
0,006
0,004
0,002
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
(B)
Control 5-CQA and CoA no enzyme overnight
5-CQA
4-CQA
3-CQA
AU
0,050
0,040
0,030
0,020
0,010
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 34

(C)

HCT 1/1 5-CQA/CoA ratio (sample 1085) and overlap of absorbance spectra of the diCQA peaks

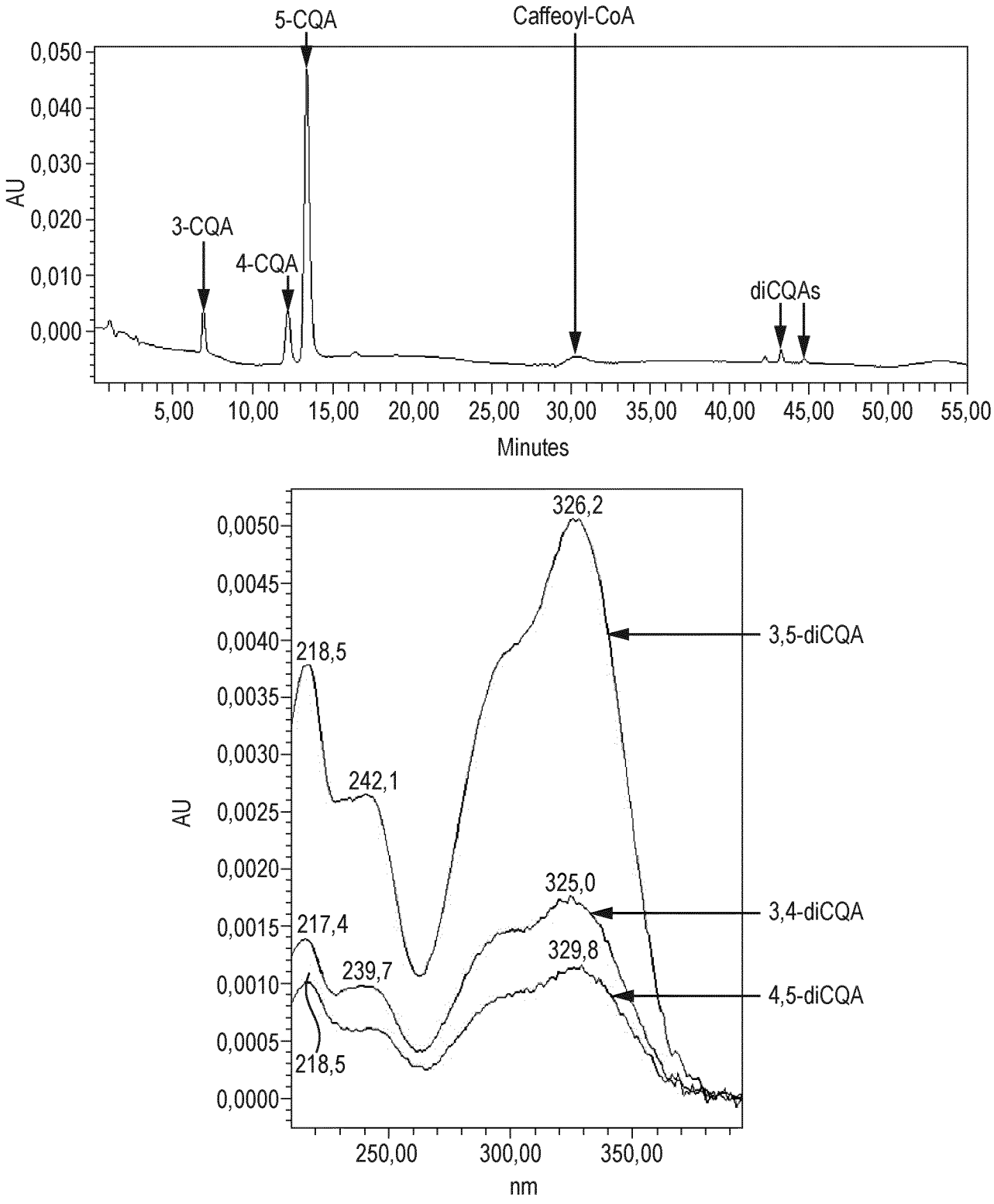


FIG. 34 Cont'd

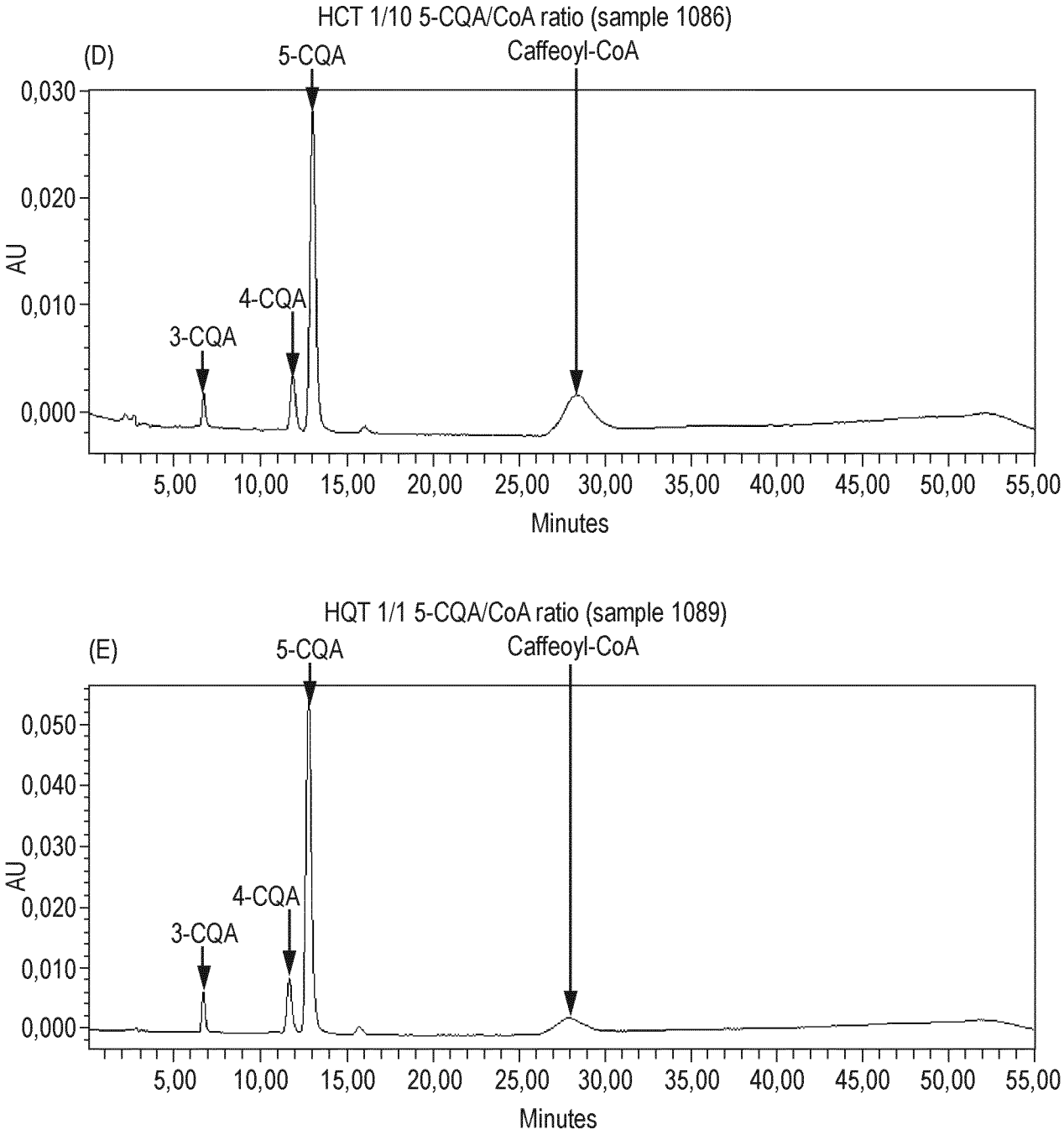

HCT 1/10 5-CQA/CoA ratio (sample 1086)
(D)
5-CQA
Caffeoyl-CoA
0,030
0,020
0,010
0,000
AU
4-CQA
3-CQA
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
HQT 1/1 5-CQA/CoA ratio (sample 1089)
(E)
5-CQA
Caffeoyl-CoA
0,050
0,040
0,030
0,020
0,010
0,000
AU
4-CQA
3-CQA
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 34 Cont'd

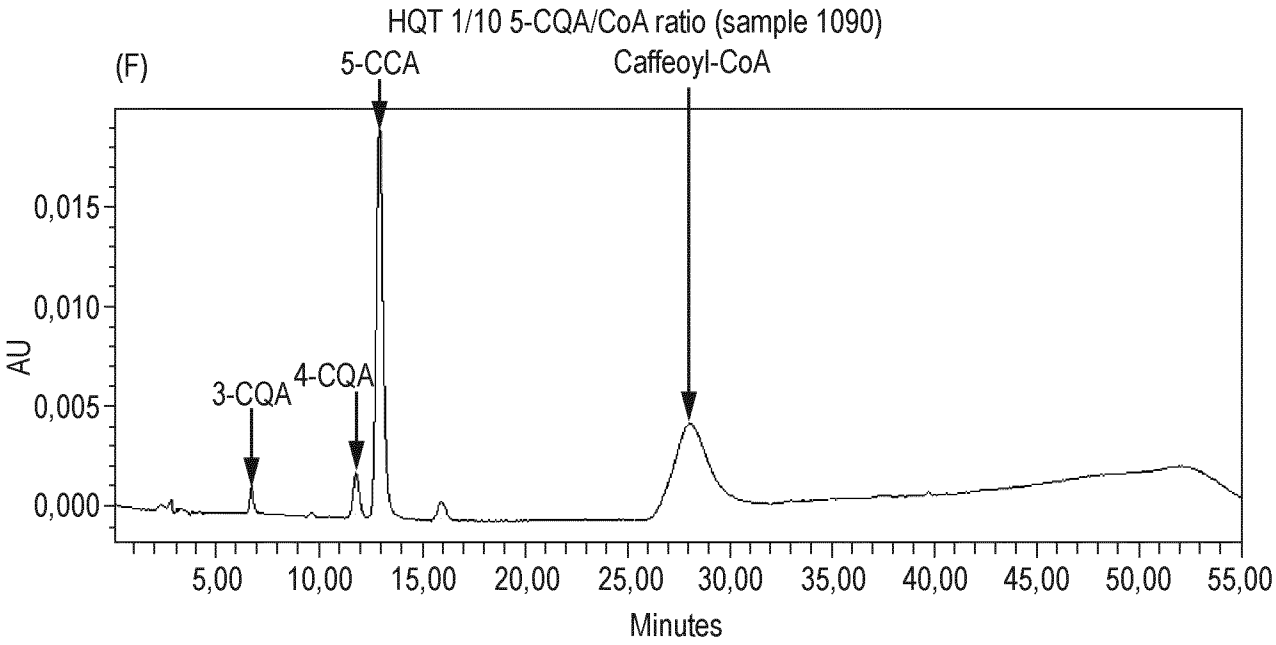
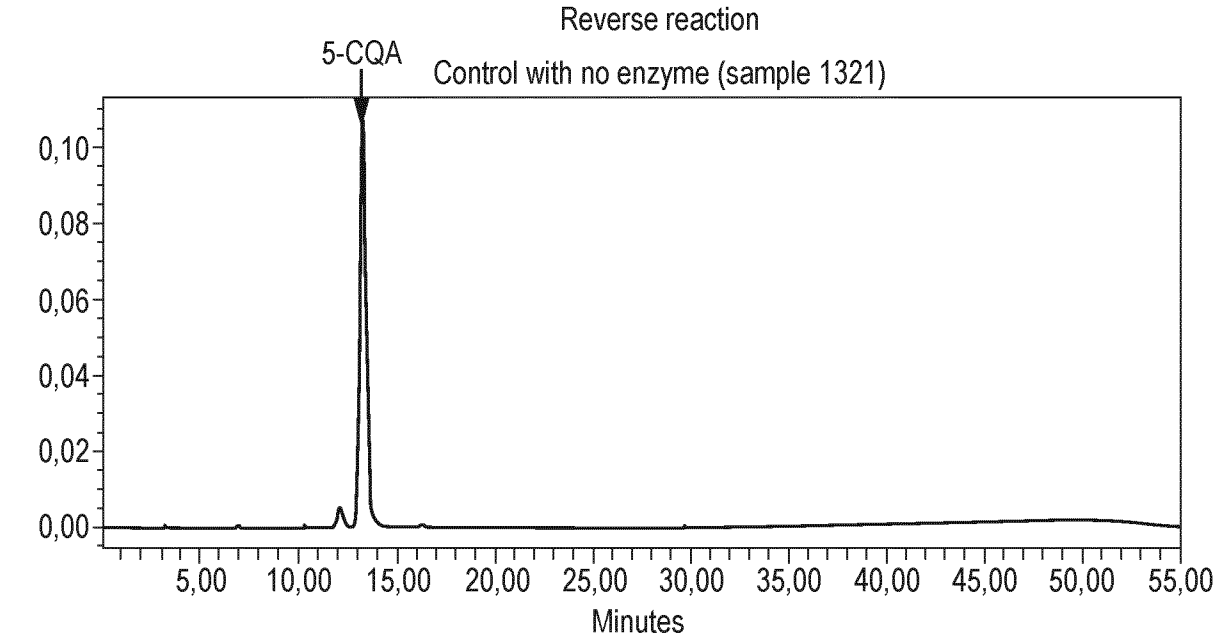

HQT 1/10 5-CQA/CoA ratio (sample 1090)
(F)
5-CCA
Caffeoyl-CoA
3-CQA
4-CQA
AU
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 34 Cont'd


Reverse reaction
5-CQA
Control with no enzyme (sample 1321)
0,10
0,08
0,06
0,04
0,02
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

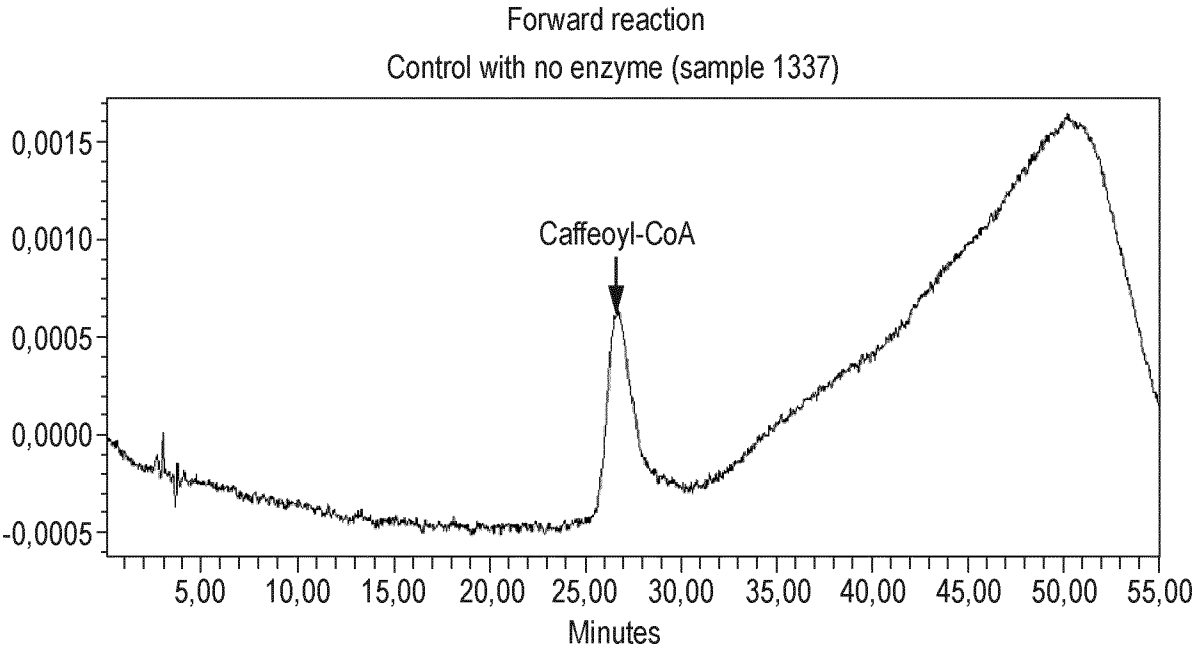

Forward reaction
Control with no enzyme (sample 1337)
0,0015
0,0010
0,0005
0,0000
-0,0005
Caffeoyl-CoA
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 35

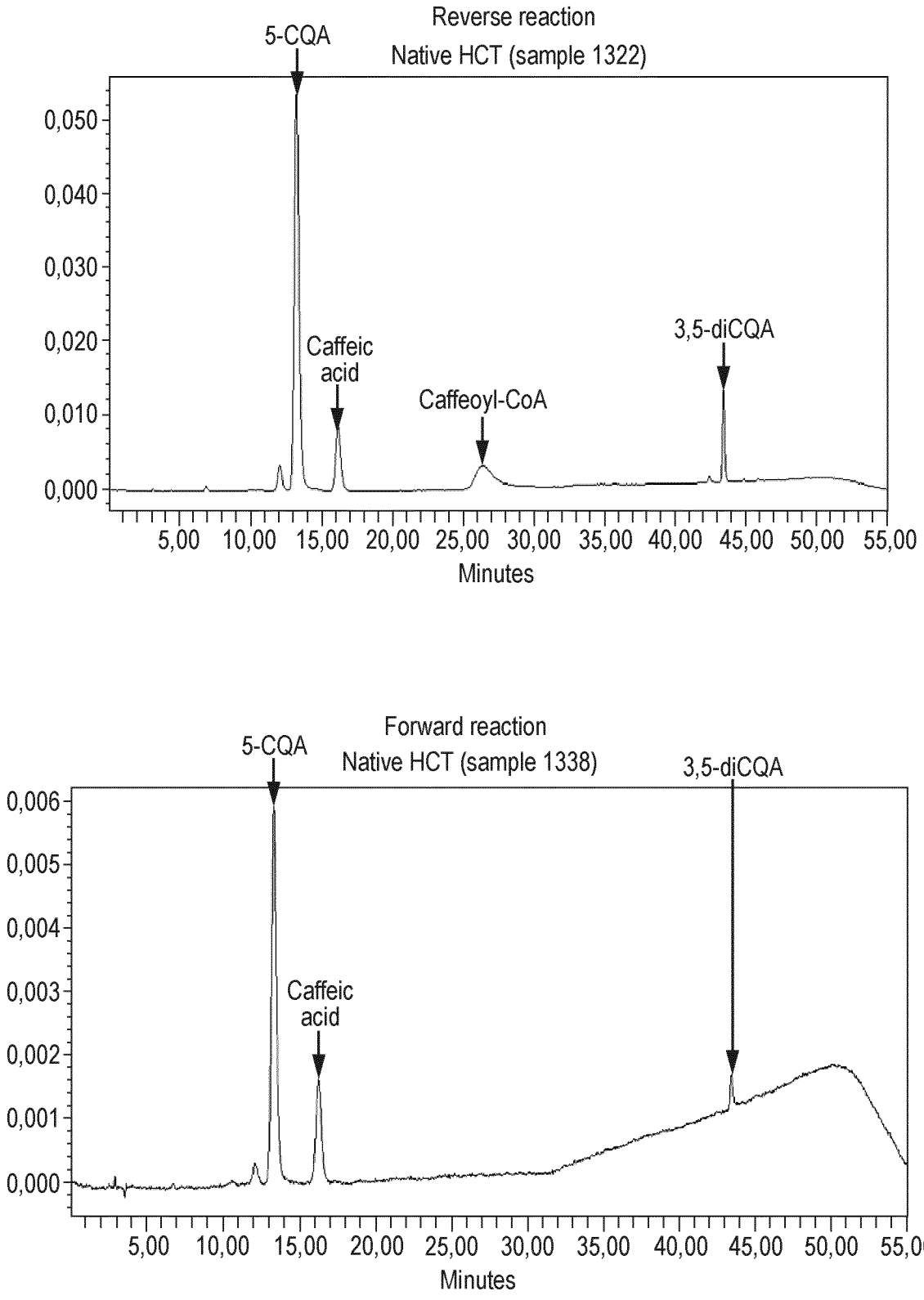
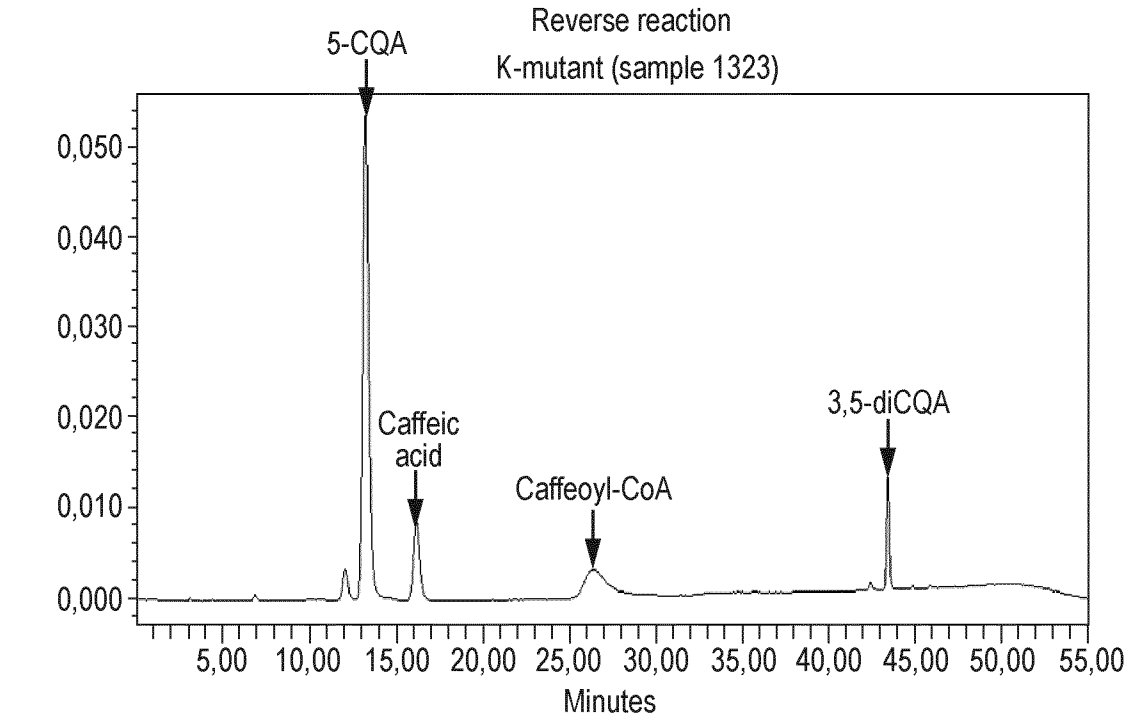

Reverse reaction
Native HCT (sample 1322)
5-CQA
Caffeic acid
Caffeoyl-CoA
3,5-diCQA
0,050
0,040
0,030
0,020
0,010
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
Forward reaction
Native HCT (sample 1338)
5-CQA
Caffeic acid
3,5-diCQA
0,006
0,005
0,004
0,003
0,002
0,001
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 35 Cont'd


Reverse reaction
K-mutant (sample 1323)
5-CQA
Caffeic acid
Caffeoyl-CoA
3,5-diCQA
0,050
0,040
0,030
0,020
0,010
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

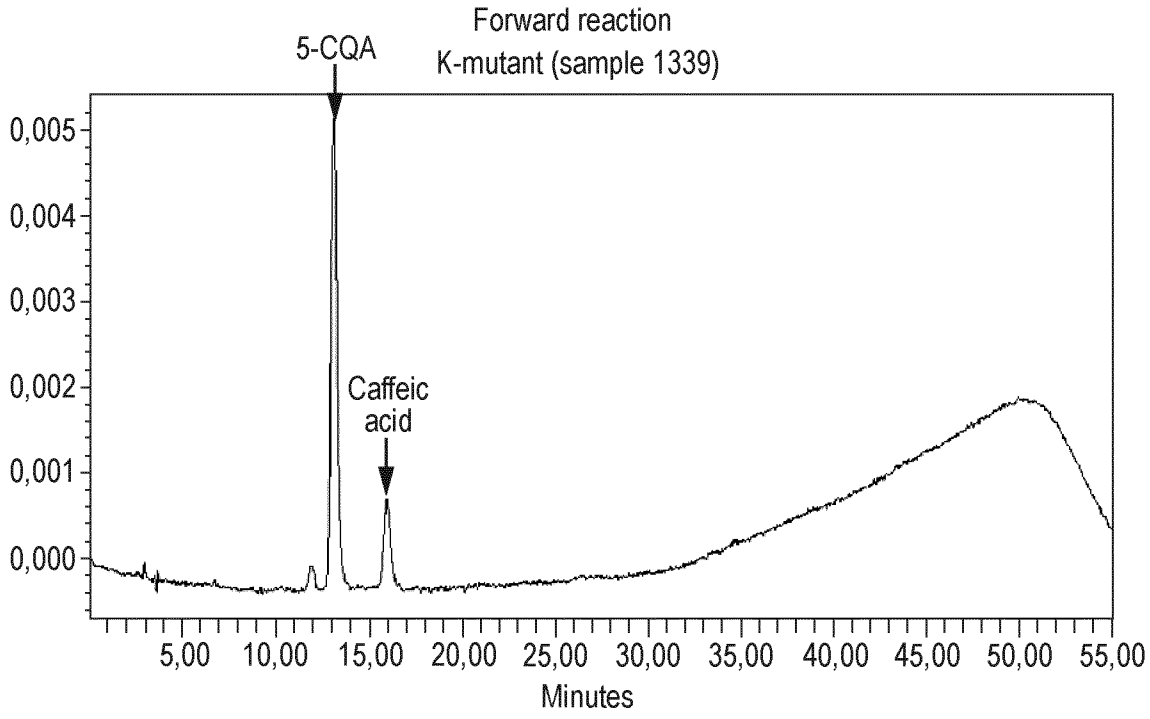
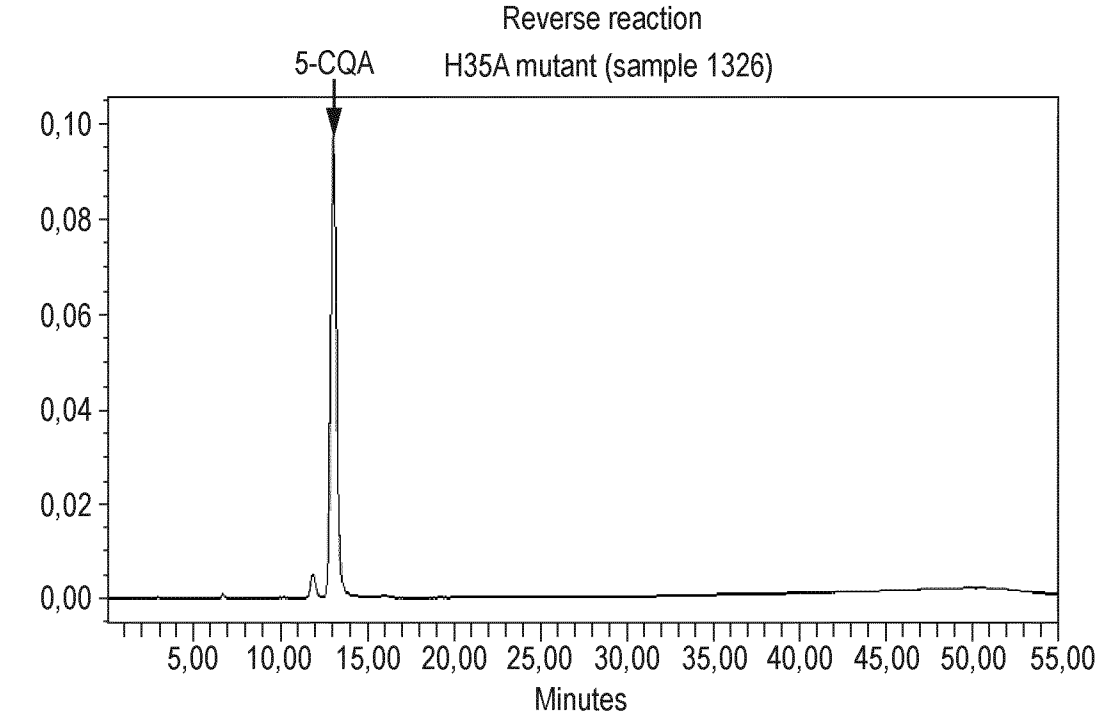

Forward reaction
K-mutant (sample 1339)
5-CQA
Caffeic acid
0,005
0,004
0,003
0,002
0,001
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 35 Cont'd


Reverse reaction
5-CQA
H35A mutant (sample 1326)
0,10
0,08
0,06
0,04
0,02
0,00
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

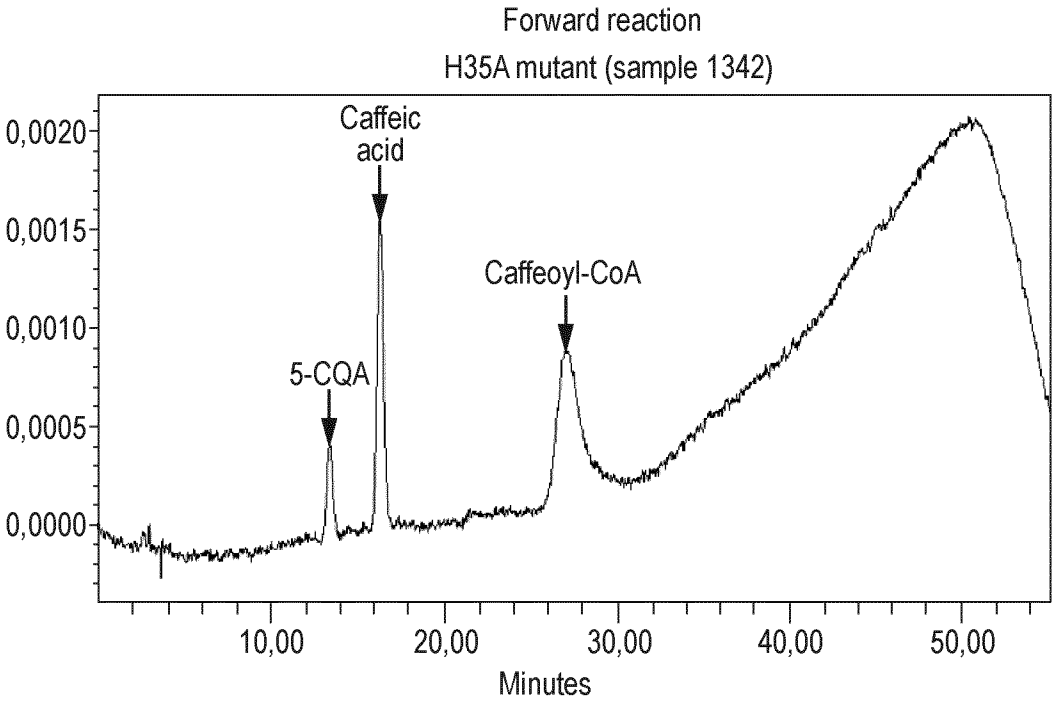
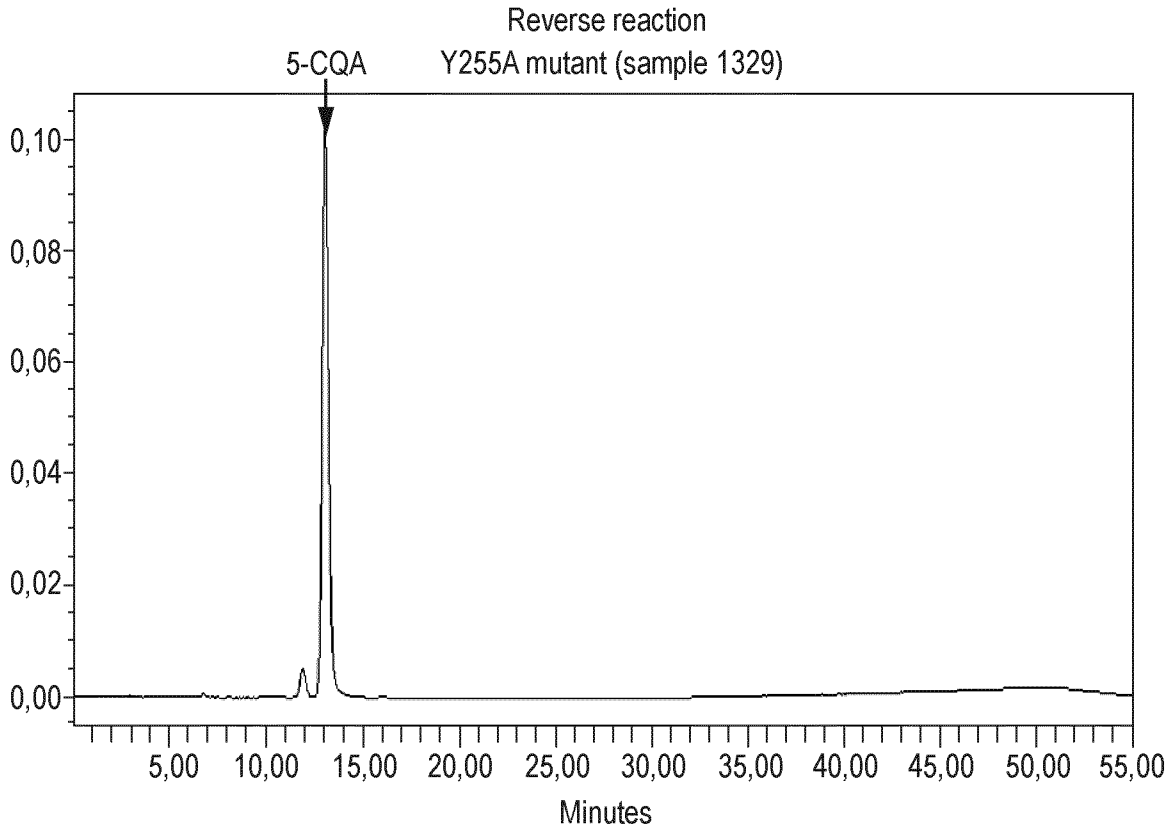

Forward reaction
H35A mutant (sample 1342)
Caffeic acid
Caffeoyl-CoA
5-CQA
0,0020
0,0015
0,0010
0,0005
0,0000
10,00 20,00 30,00 40,00 50,00
Minutes

FIG. 35 Cont'd


Reverse reaction
5-CQA
Y255A mutant (sample 1329)
0,10
0,08
0,06
0,04
0,02
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

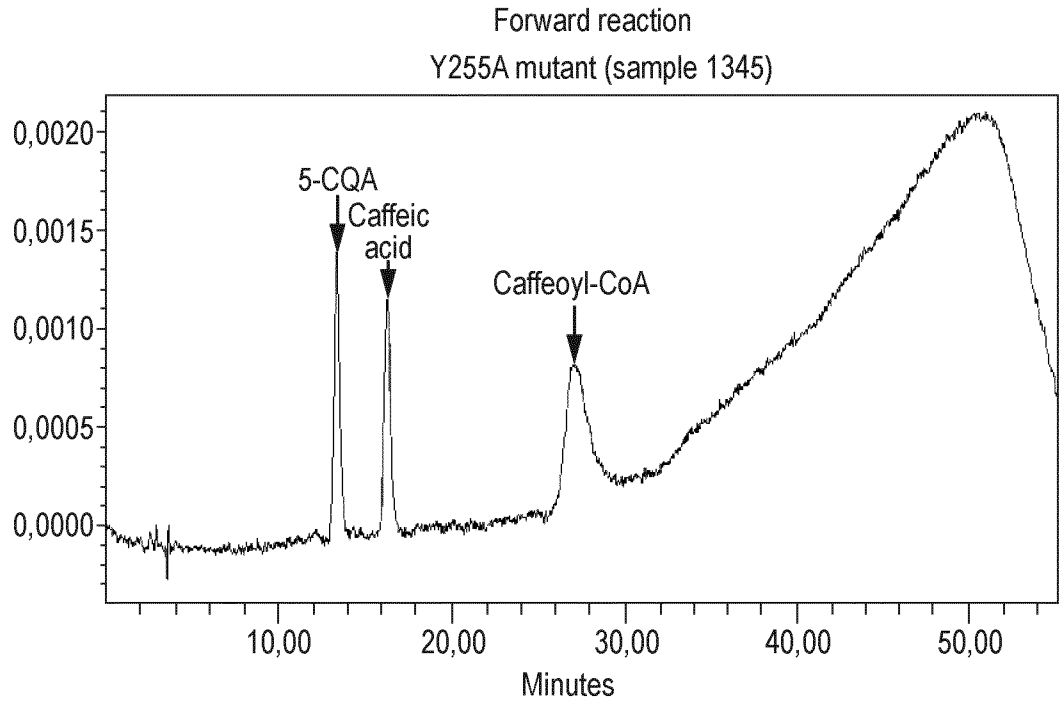
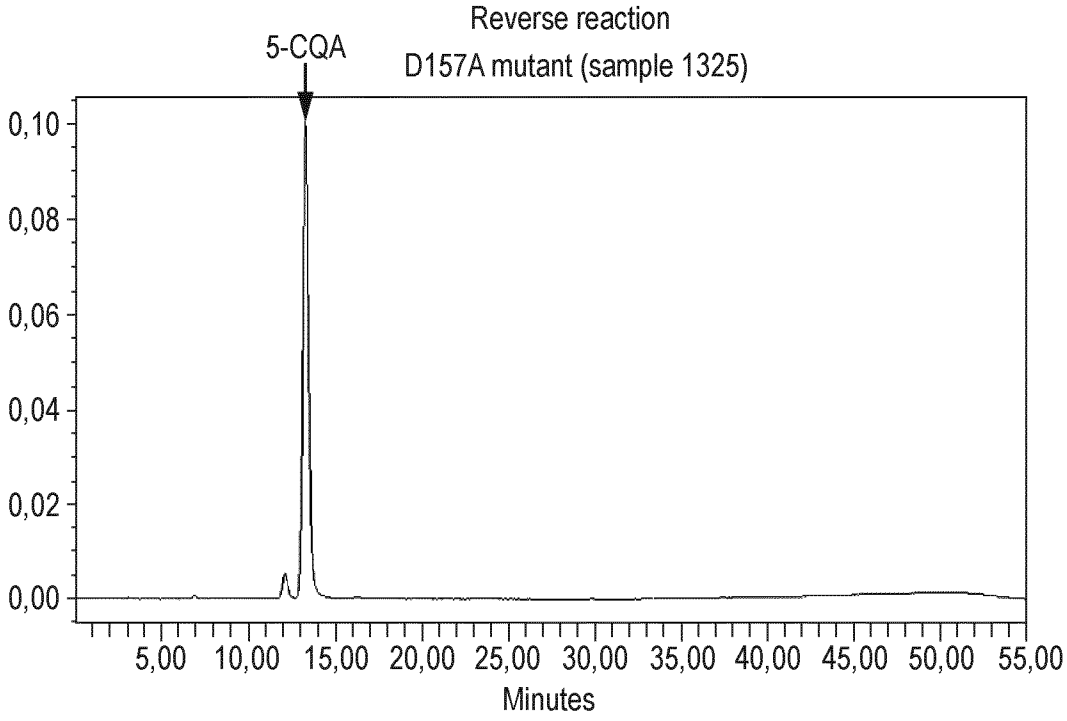

Forward reaction
Y255A mutant (sample 1345)
0,0020
0,0015
0,0010
0,0005
0,0000
5-CQA
Caffeic acid
Caffeoyl-CoA
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 35 Cont'd


Reverse reaction
5-CQA
D157A mutant (sample 1325)
0,10
0,08
0,06
0,04
0,02
0,00
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

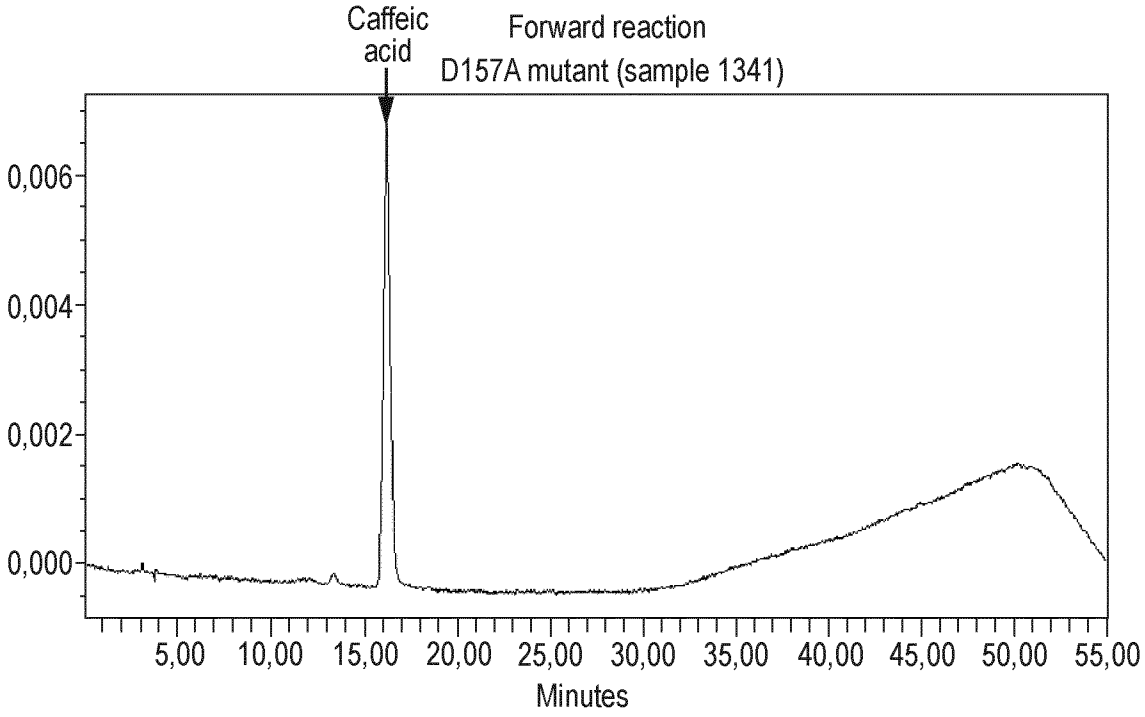
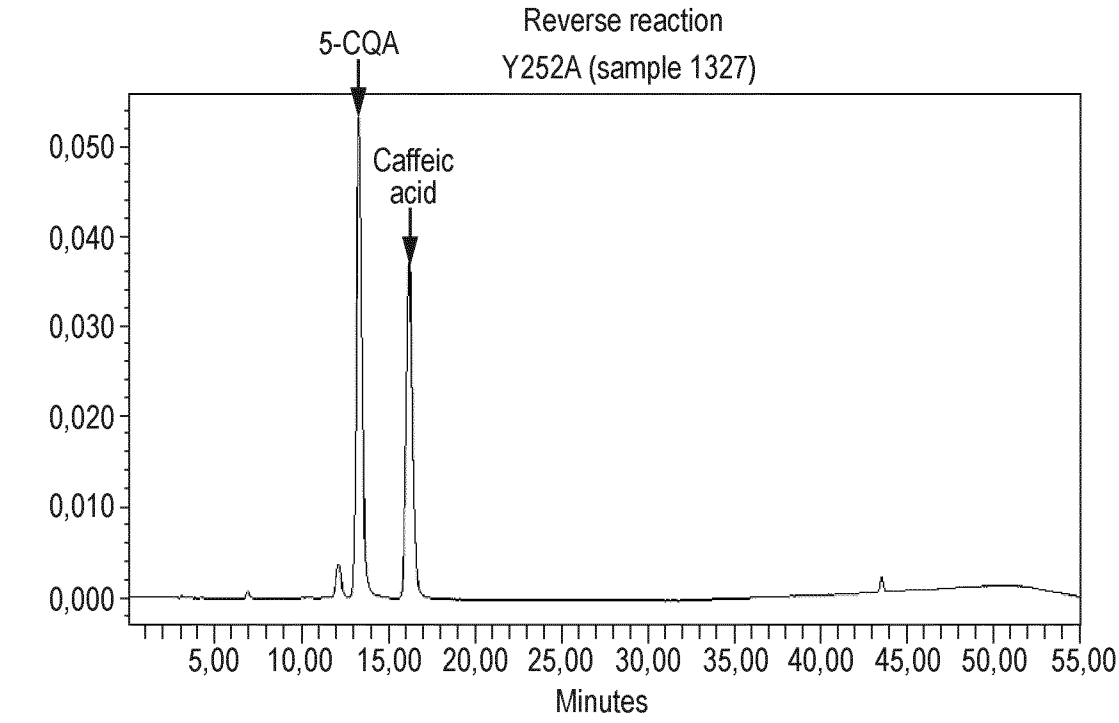

Caffeic acid
Forward reaction
D157A mutant (sample 1341)
0,006
0,004
0,002
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 35 Cont'd


Reverse reaction
Y252A (sample 1327)
5-CQA
Caffeic acid
0,050
0,040
0,030
0,020
0,010
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

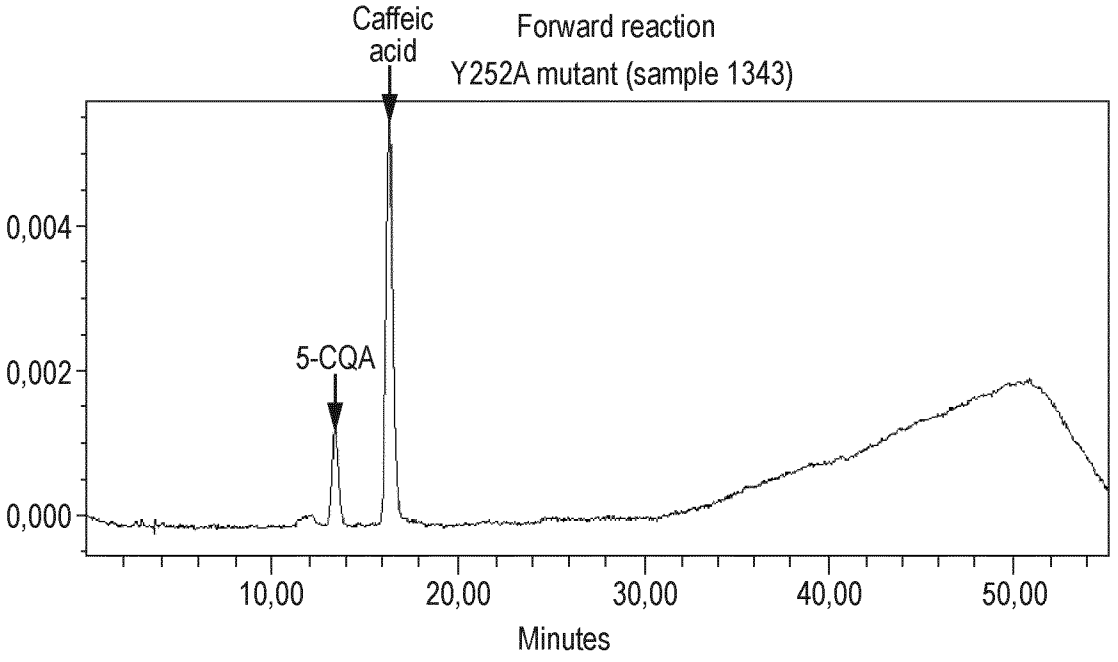
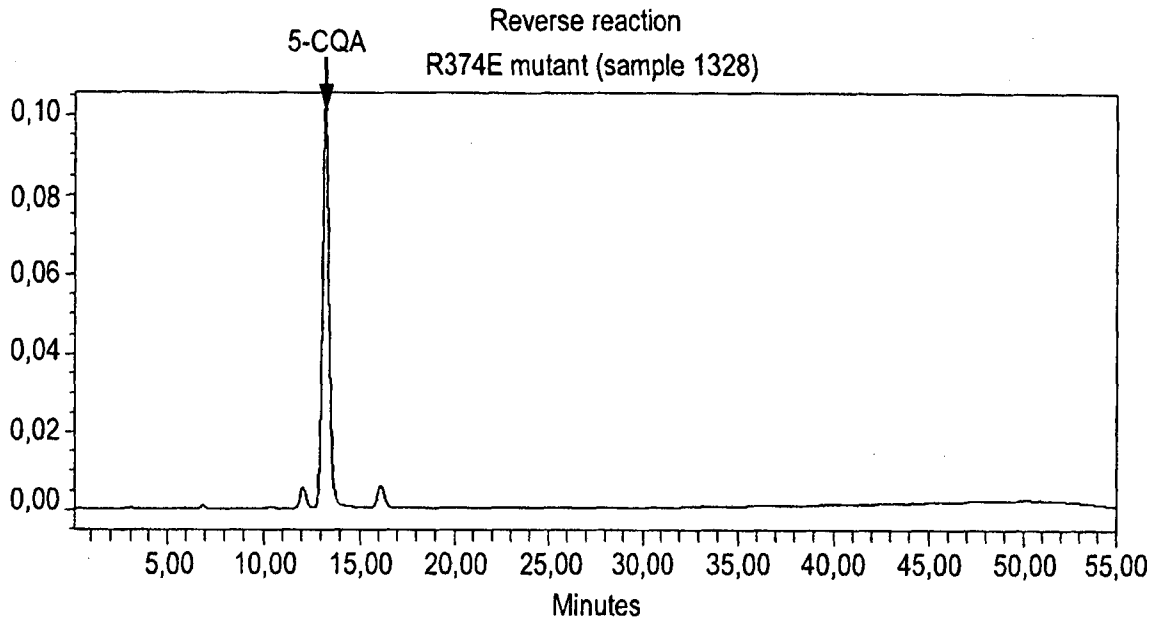

Caffeic acid
Forward reaction
Y252A mutant (sample 1343)
5-CQA
0,004
0,002
0,000
10,00 20,00 30,00 40,00 50,00
Minutes

FIG. 35 Cont'd


5-CQA
Reverse reaction
R374E mutant (sample 1328)
0,10
0,08
0,06
0,04
0,02
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

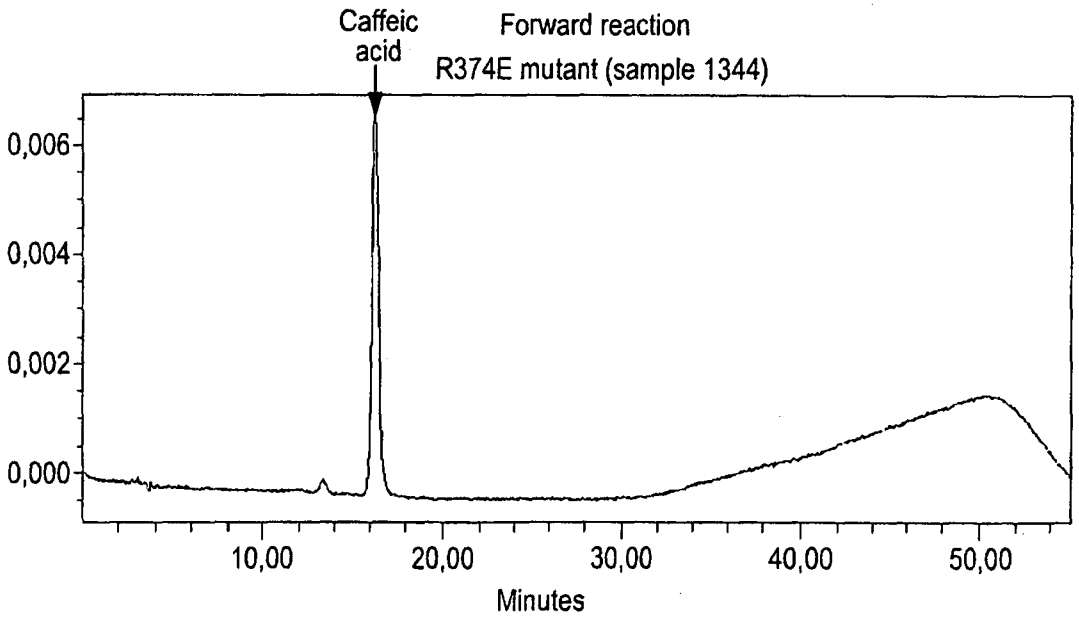
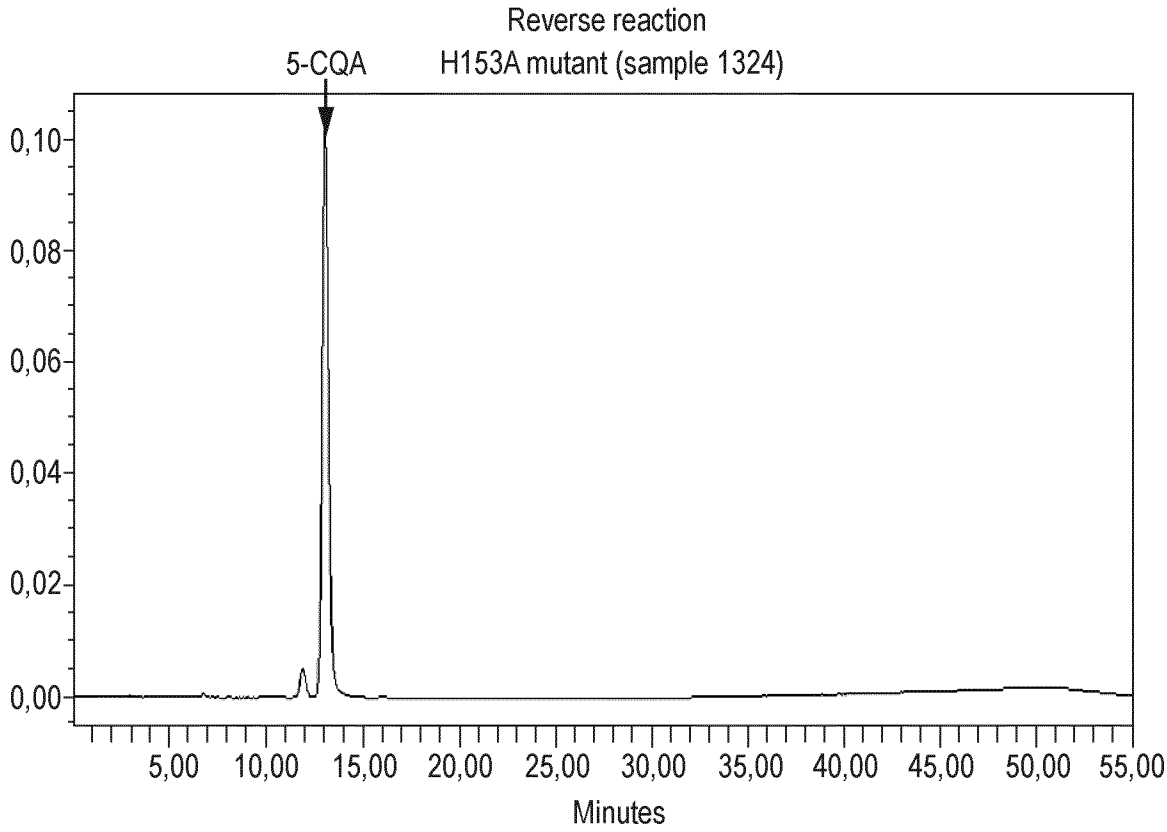

Caffeic acid
Forward reaction
R374E mutant (sample 1344)
0,006
0,004
0,002
0,000
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 35 Cont'd


Reverse reaction
5-CQA
H153A mutant (sample 1324)
0,10
0,08
0,06
0,04
0,02
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

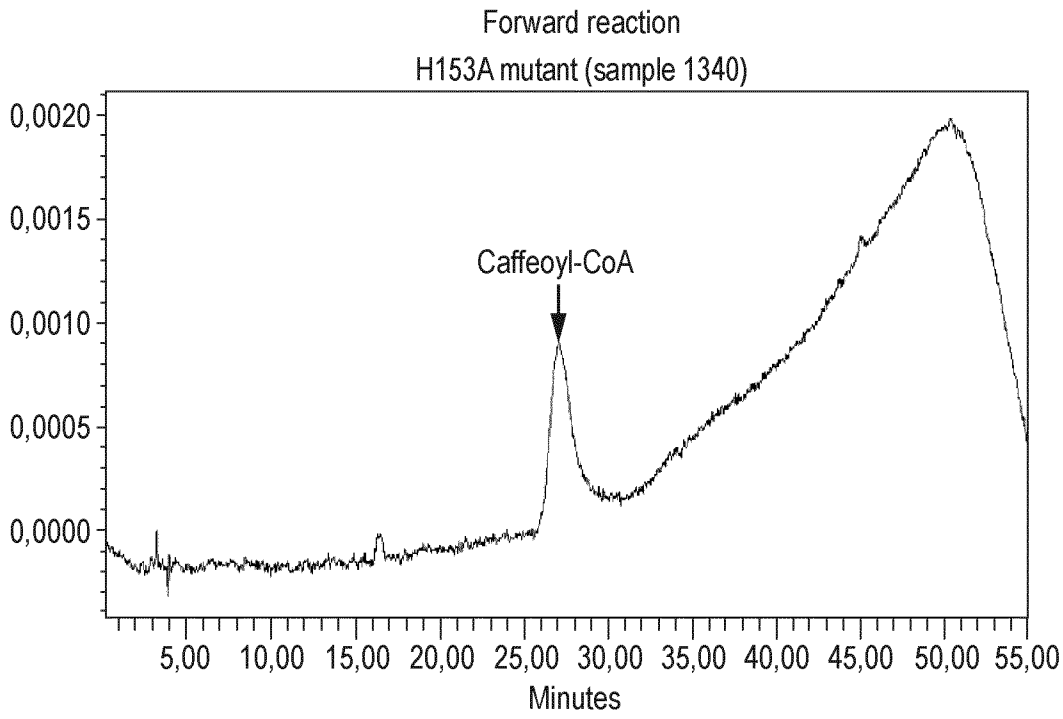

Forward reaction
H153A mutant (sample 1340)
0,0020
0,0015
0,0010
0,0005
0,0000
Caffeoyl-CoA
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 35 Cont'd

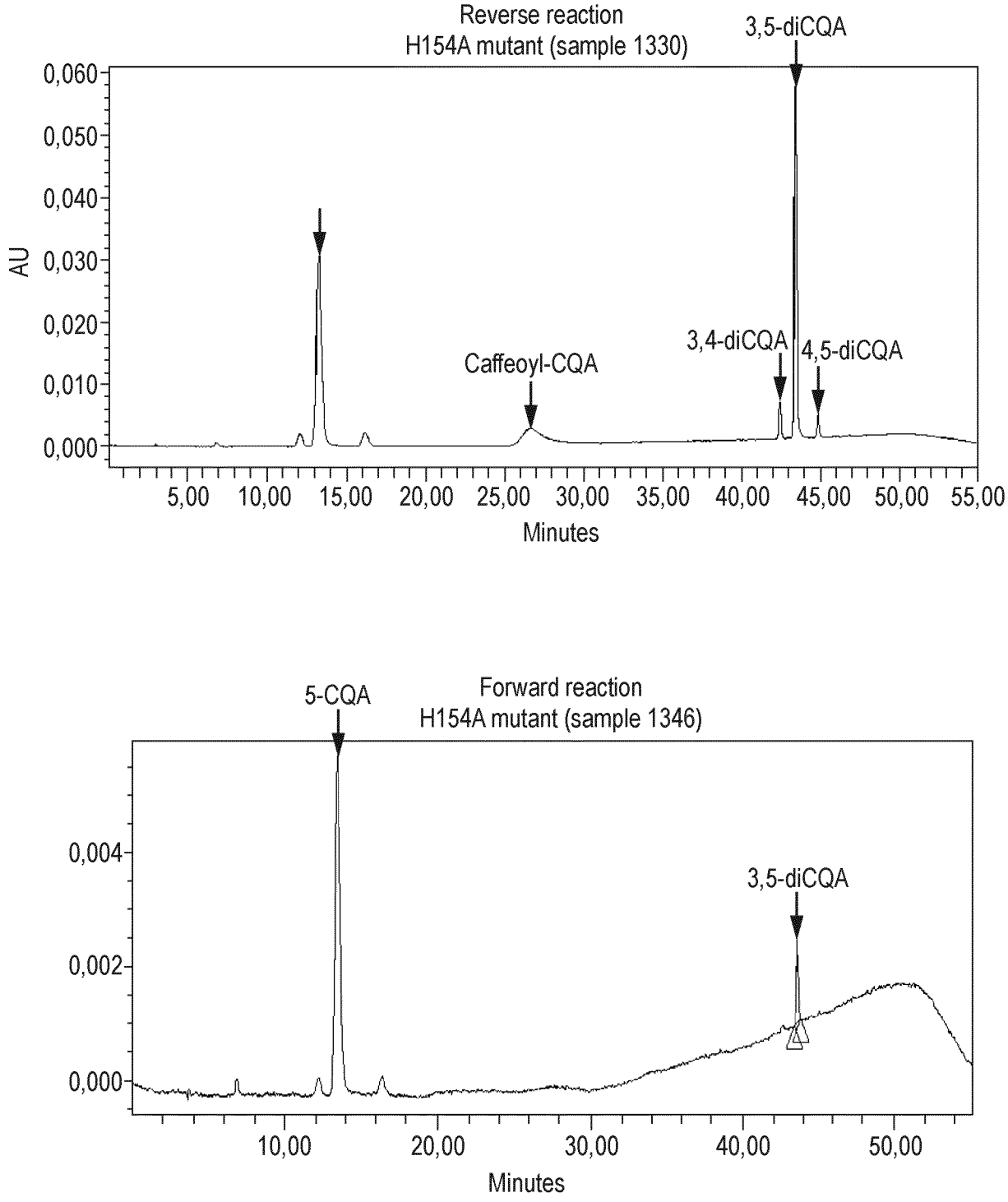

Reverse reaction
H154A mutant (sample 1330)
3,5-diCQA
0,060
0,050
0,040
0,030
0,020
0,010
0,000
AU
Caffeoyl-CQA
3,4-diCQA
4,5-diCQA
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
5-CQA
Forward reaction
H154A mutant (sample 1346)
0,004
0,002
0,000
3,5-diCQA
10,00
20,00
30,00
40,00
50,00
Minutes

FIG. 35 Cont'd

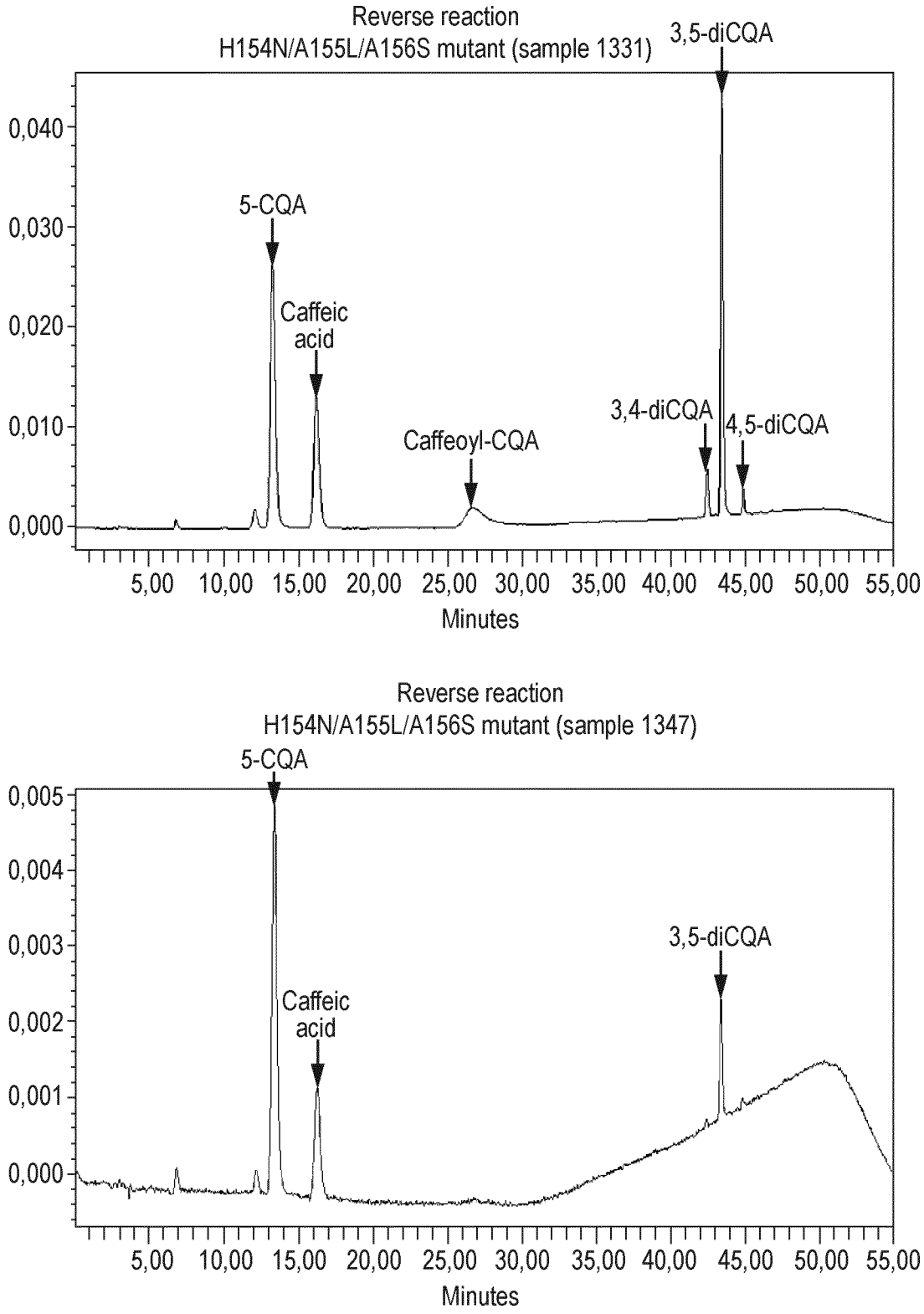

Reverse reaction
H154N/A155L/A156S mutant (sample 1331)
3,5-diCQA
5-CQA
Caffeic acid
3,4-diCQA
4,5-diCQA
Caffeoyl-CQA
0,040
0,030
0,020
0,010
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
Reverse reaction
H154N/A155L/A156S mutant (sample 1347)
5-CQA
3,5-diCQA
Caffeic acid
0,005
0,004
0,003
0,002
0,001
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 35 Cont'd

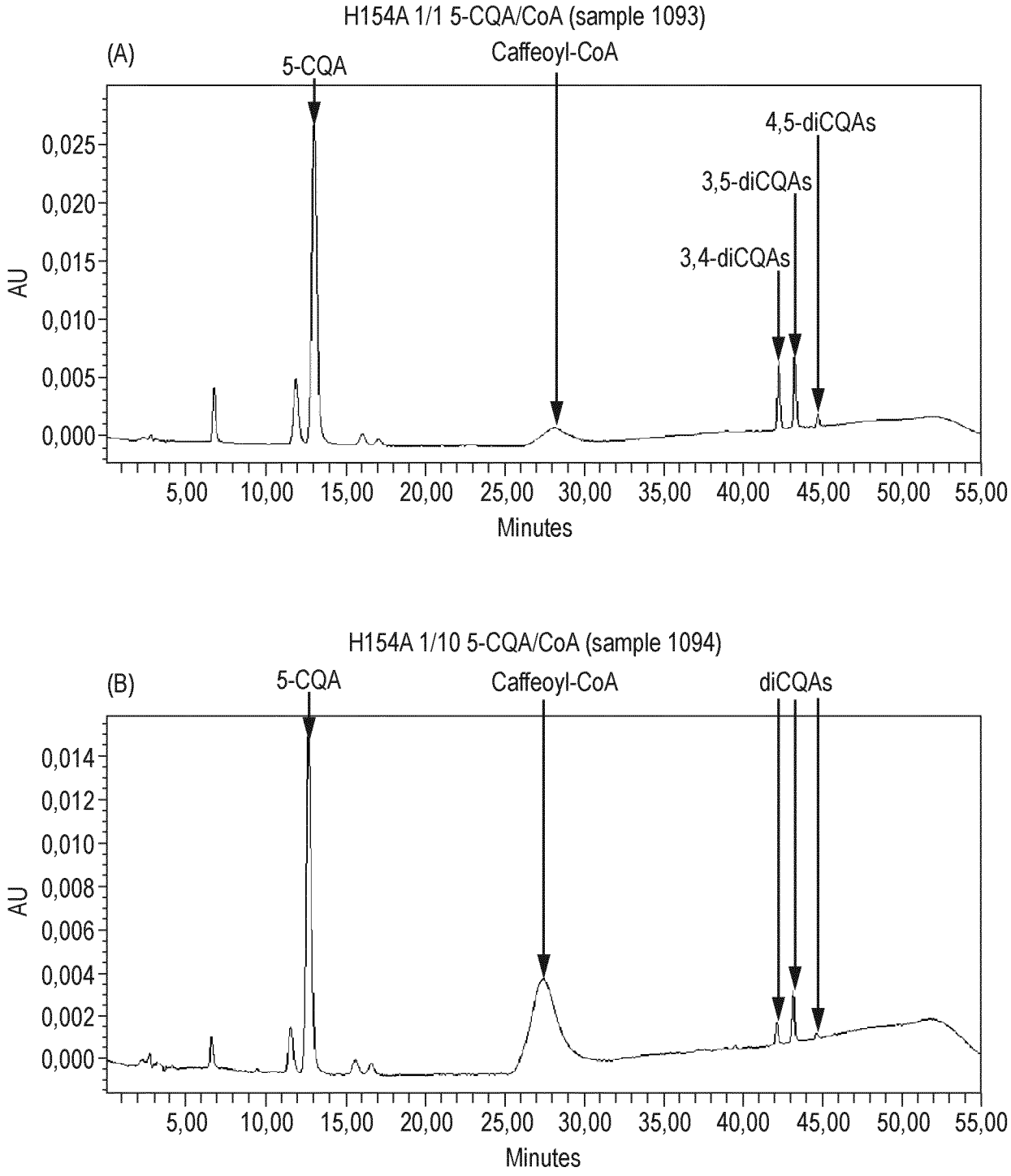

H154A 1/1 5-CQA/CoA (sample 1093)
(A)
5-CQA
Caffeoyl-CoA
4,5-diCQAs
3,5-diCQAs
3,4-diCQAs
AU
0,025
0,020
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
H154A 1/10 5-CQA/CoA (sample 1094)
(B)
5-CQA
Caffeoyl-CoA
diCQAs
AU
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 36

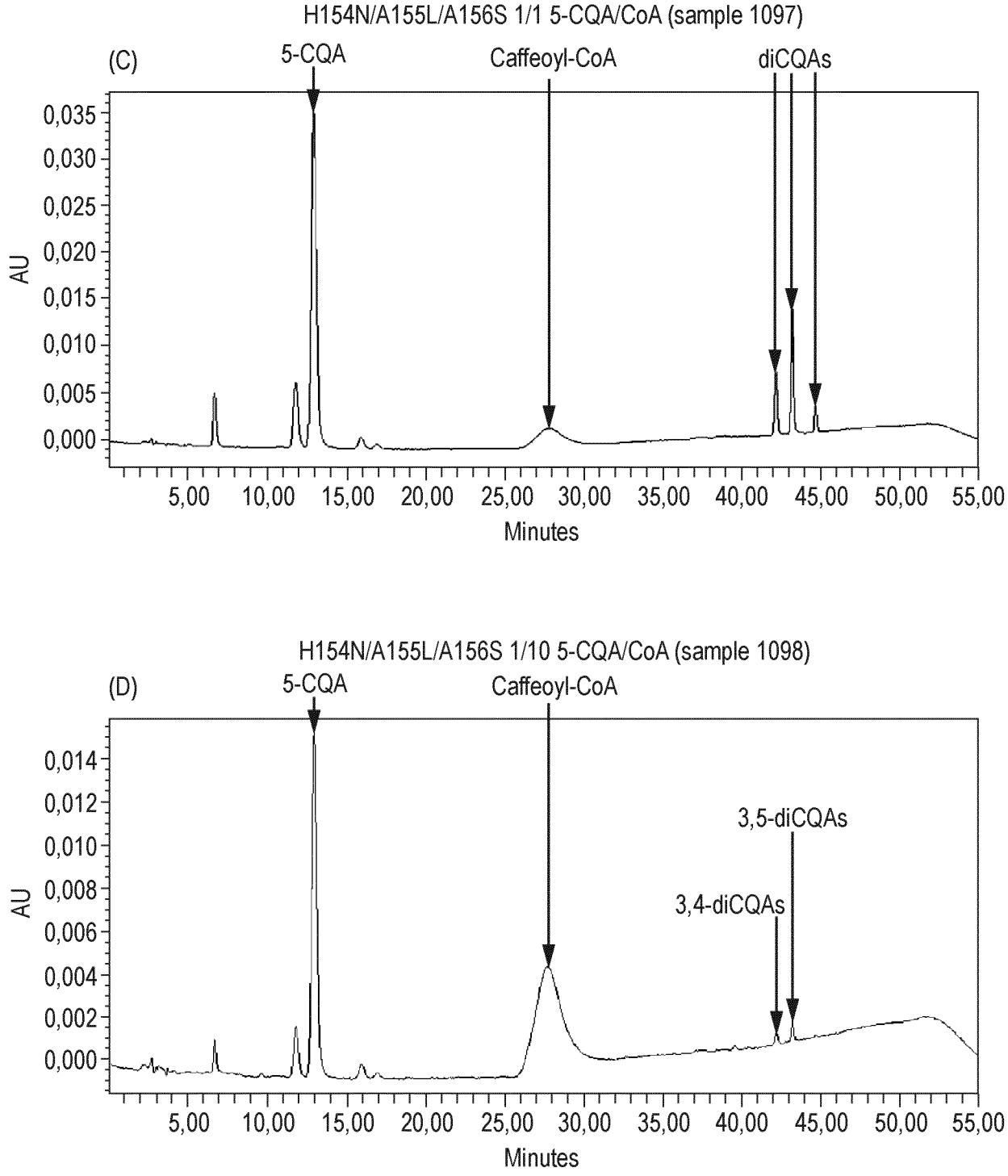

H154N/A155L/A156S 1/1 5-CQA/CoA (sample 1097)
(C)
5-CQA
Caffeoyl-CoA
diCQAs
AU
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
H154N/A155L/A156S 1/10 5-CQA/CoA (sample 1098)
(D)
5-CQA
Caffeoyl-CoA
3,5-diCQAs
3,4-diCQAs
AU
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 36 Cont'd

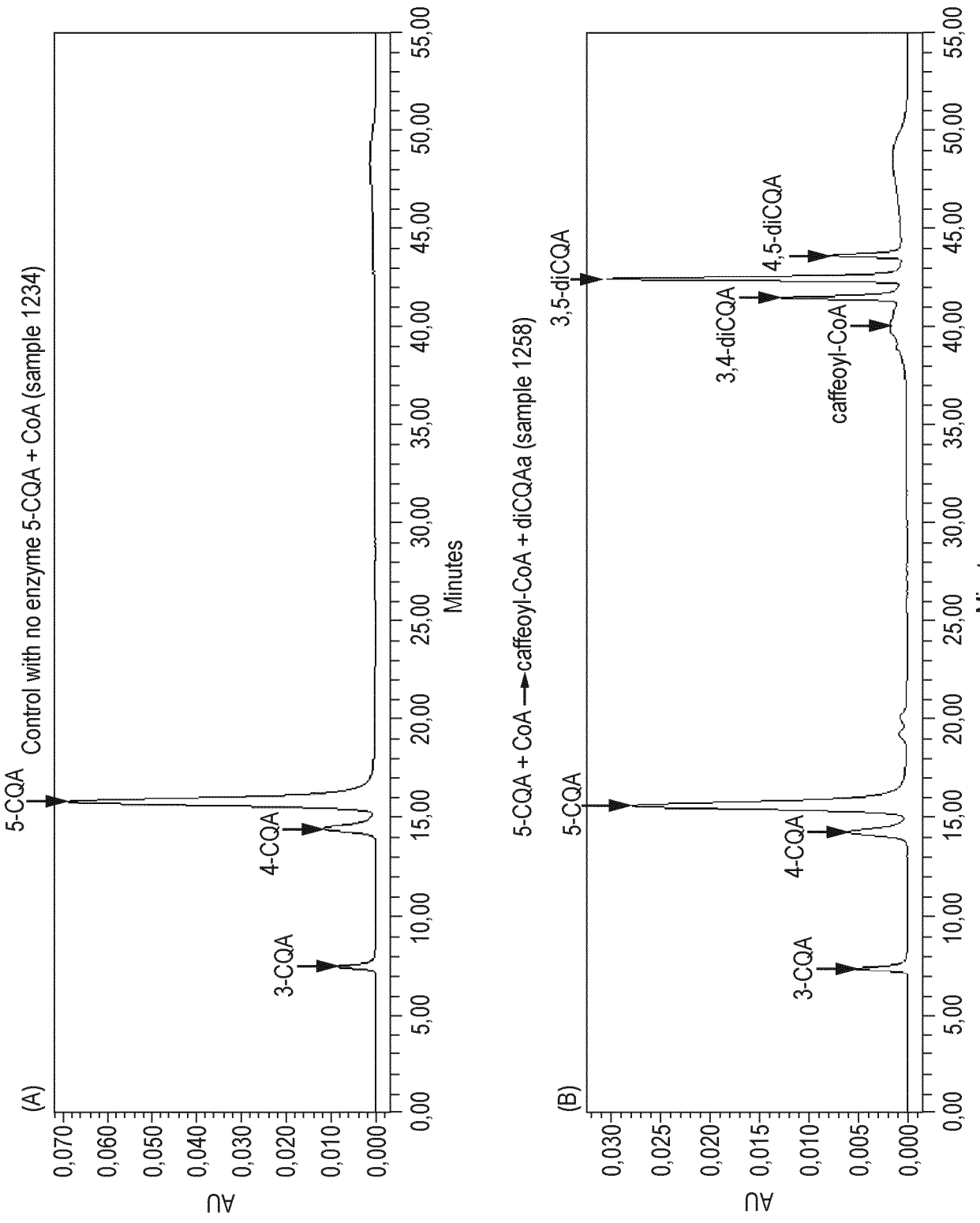

(A)
Control with no enzyme 5-CQA + CoA (sample 1234)
5-CQA
4-CQA
3-CQA
AU
Minutes
(B)
5-CQA + CoA → caffeoyl-CoA + diCQAa (sample 1258)
3,5-diCQA
4,5-diCQA
3,4-diCQA
caffeoyl-CoA
5-CQA
4-CQA
3-CQA
AU
Minutes

FIG. 37

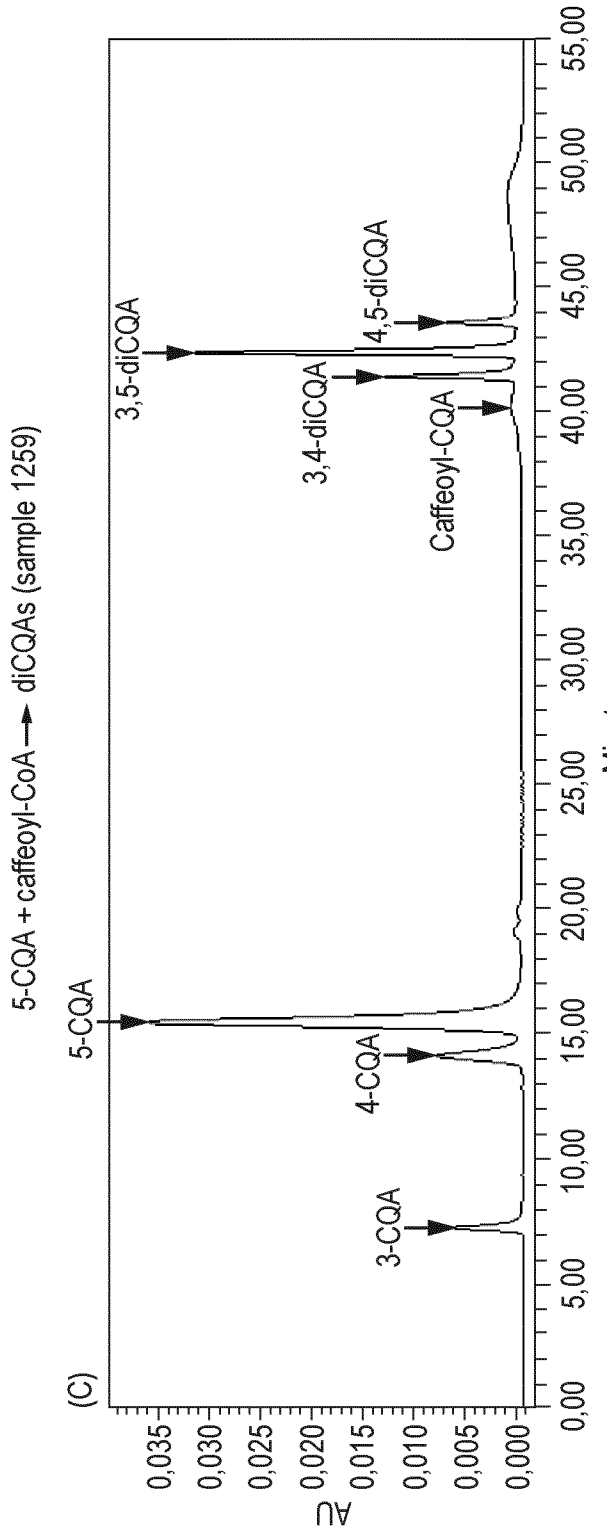

(C)
5-CQA + caffeoyl-CoA → diCQAs (sample 1259)
5-CQA
4-CQA
3-CQA
3,5-diCQA
3,4-diCQA
Caffeoyl-CQA
4,5-diCQA
AU
0,000
0,005
0,010
0,015
0,020
0,025
0,030
0,035
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 37 Cont'd

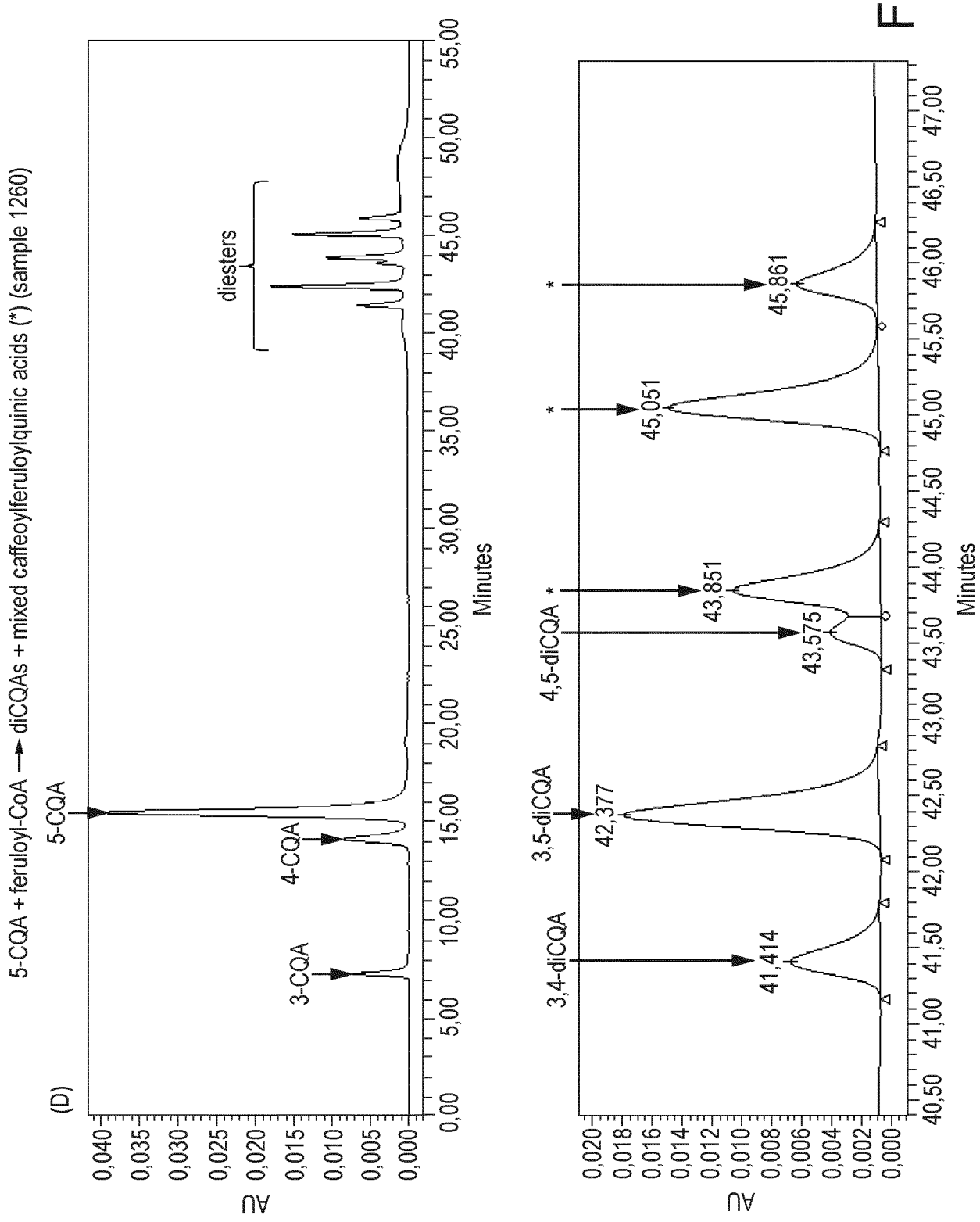

(D)
5-CQA + feruloyl-CoA → diCQAs + mixed caffeoylferuloylquinic acids (*) (sample 1260)
5-CQA
4-CQA
3-CQA
diesters
AU
Minutes
3,4-diCQA
3,5-diCQA
4,5-diCQA
41,414
42,377
43,575
43,851
45,051
45,861

FIG. 37 Cont'd

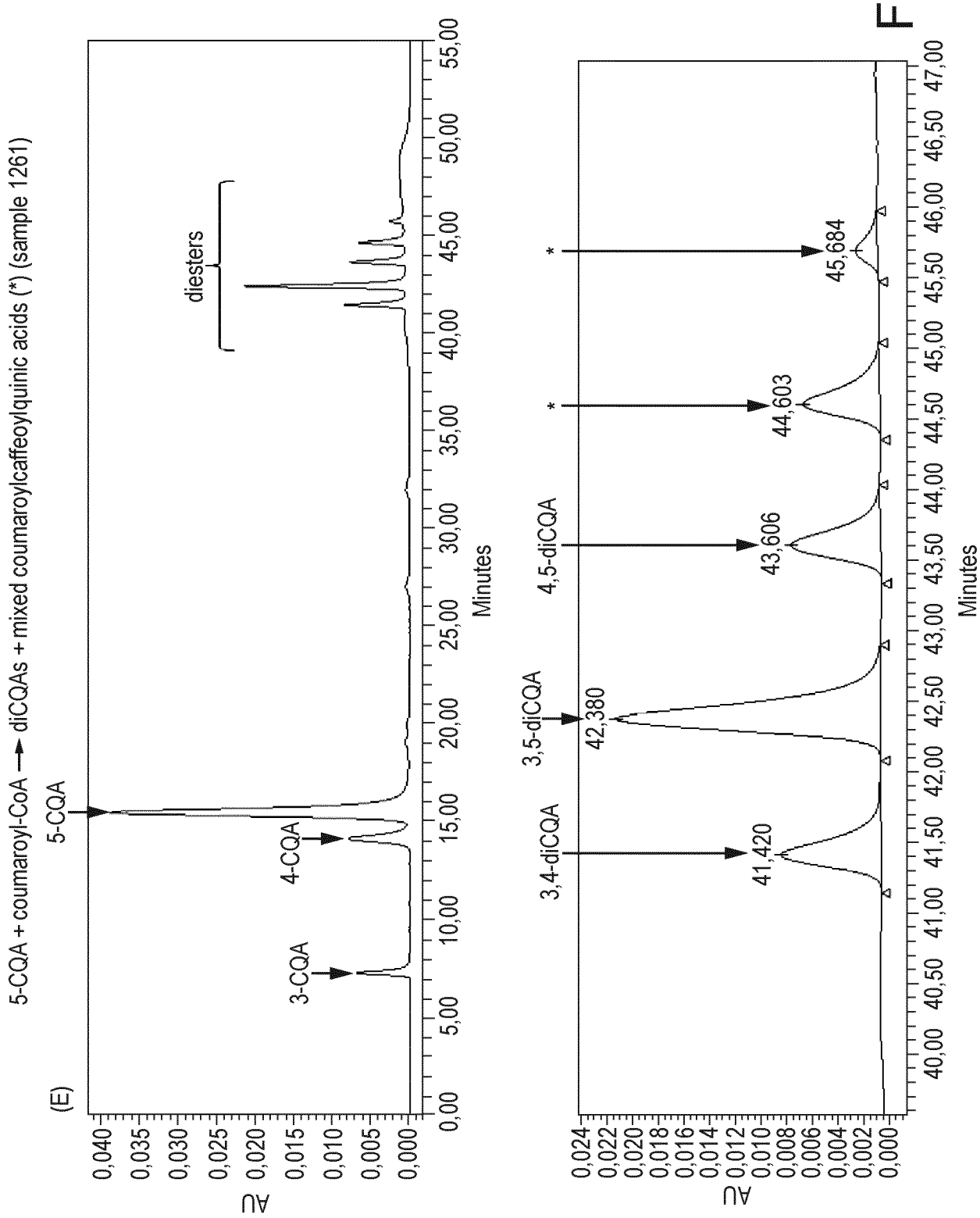

5-CQA + coumaroyl-CoA → diCQAs + mixed coumaroylcaffeoylquinic acids (*) (sample 1261)
(E)
5-CQA
4-CQA
3-CQA
diesters
AU
Minutes
3,4-diCQA
3,5-diCQA
4,5-diCQA
41,420
42,380
43,606
44,603
45,684
*

FIG. 37 Cont'd

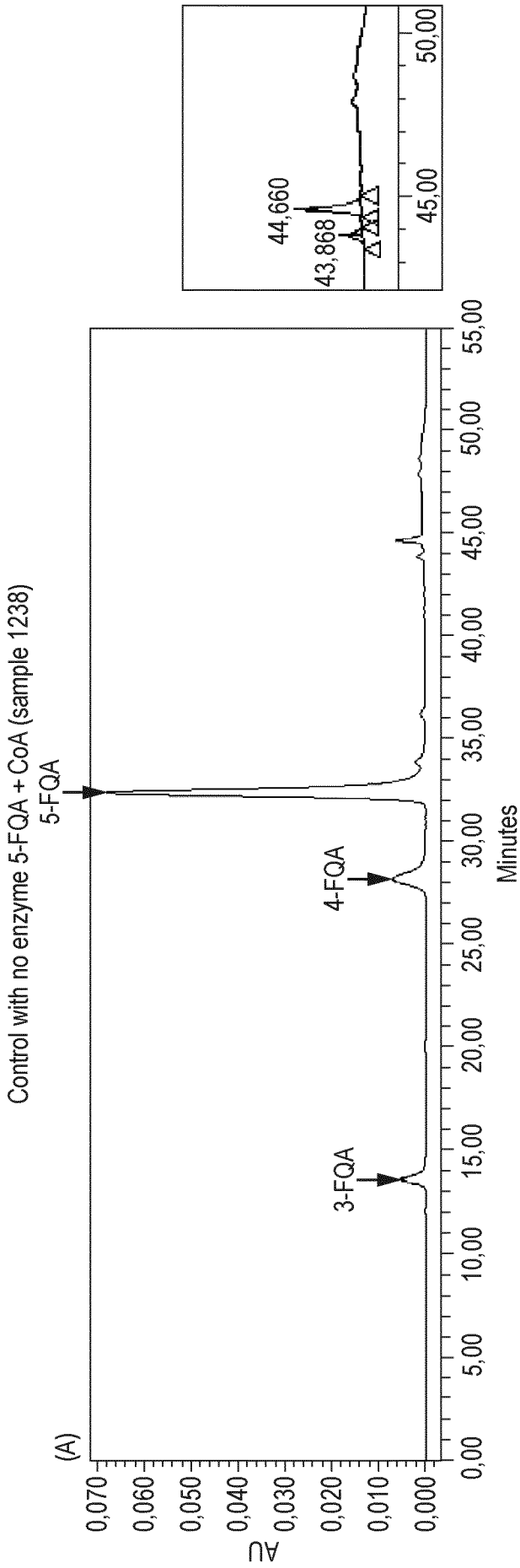

Control with no enzyme 5-FQA + CoA (sample 1238)
(A)
AU
Minutes
5-FQA
4-FQA
3-FQA
44,660
43,868
0,000
0,010
0,020
0,030
0,040
0,050
0,060
0,070
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00

FIG. 38

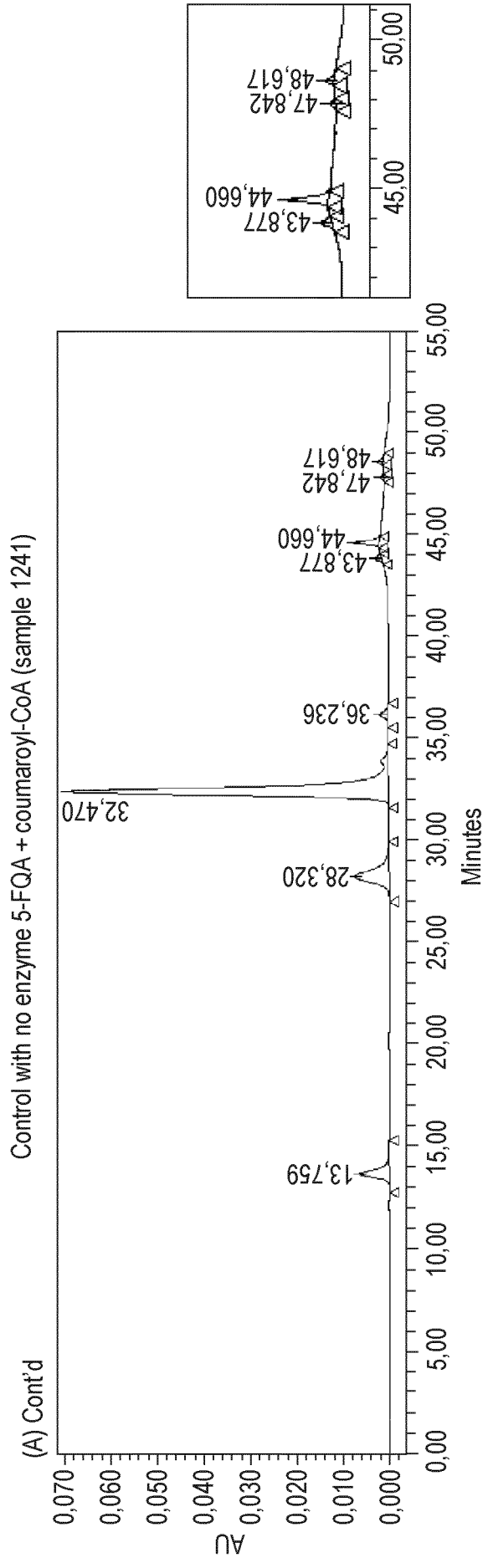
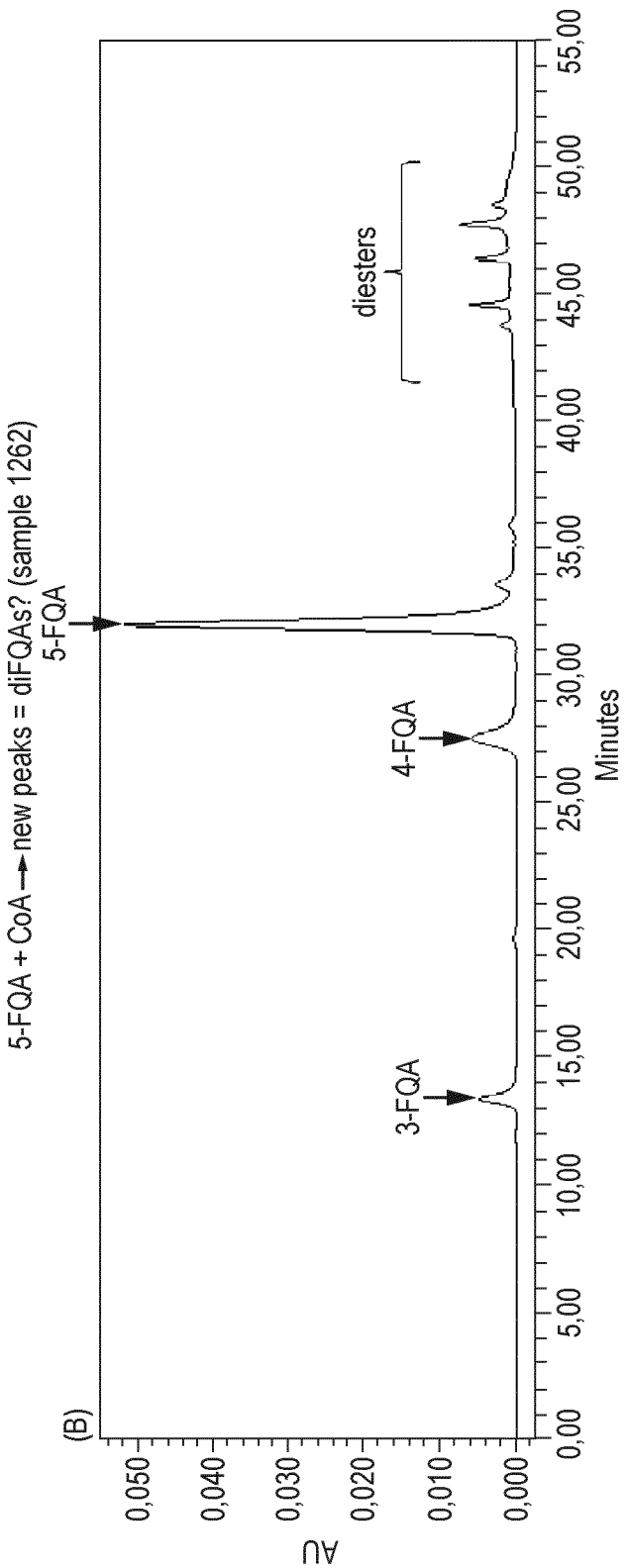

(A) Cont'd
Control with no enzyme 5-FQA + coumaroyl-CoA (sample 1241)
AU
0,000
0,010
0,020
0,030
0,040
0,050
0,060
0,070
Minutes
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
13,759
28,320
32,470
36,236
43,877
44,660
47,842
48,617
45,00
50,00

FIG. 38 Cont'd


(B)
5-FQA + CoA → new peaks = diFQAs? (sample 1262)
5-FQA
3-FQA
4-FQA
diesters
AU
Minutes
0,000
0,010
0,020
0,030
0,040
0,050
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00

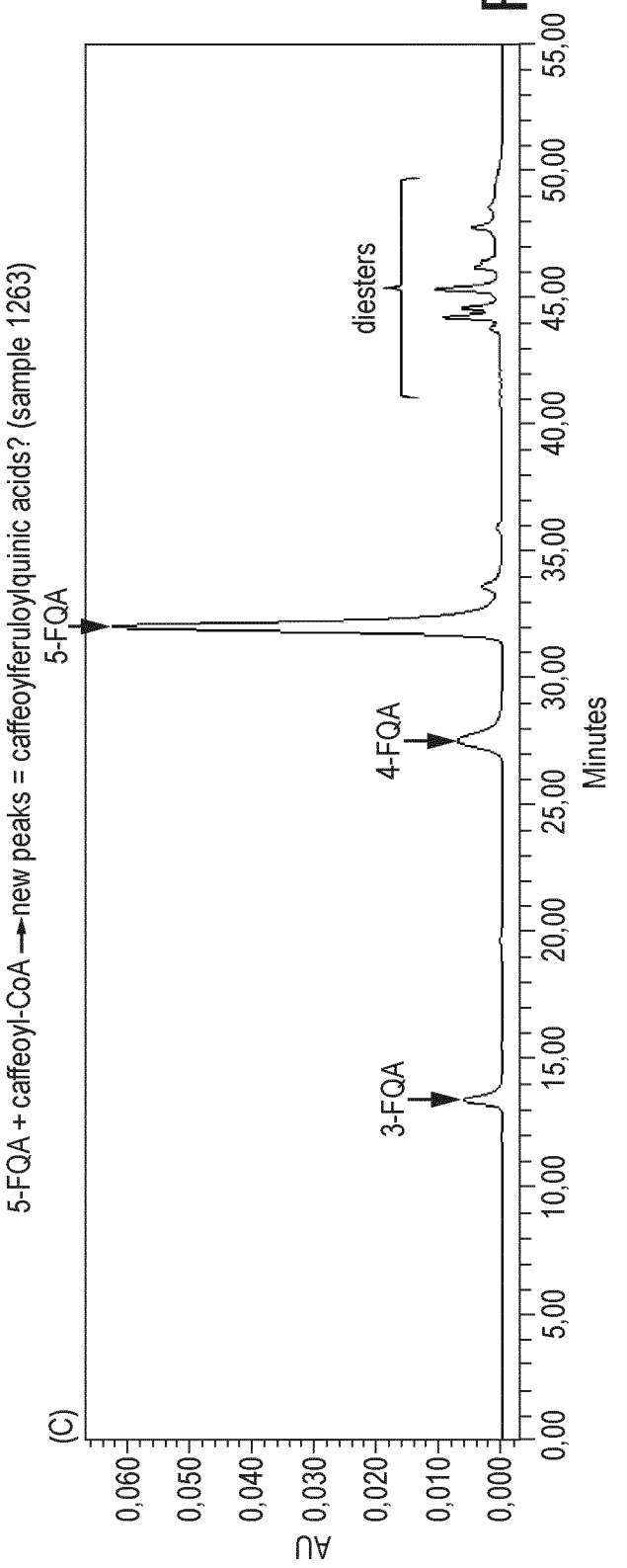

(C)
5-FQA + caffeoyl-CoA → new peaks = caffeoylferuloylquinic acids? (sample 1263)
5-FQA
3-FQA
4-FQA
diesters
AU
Minutes
0,000
0,010
0,020
0,030
0,040
0,050
0,060
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00

FIG. 38 Cont'd

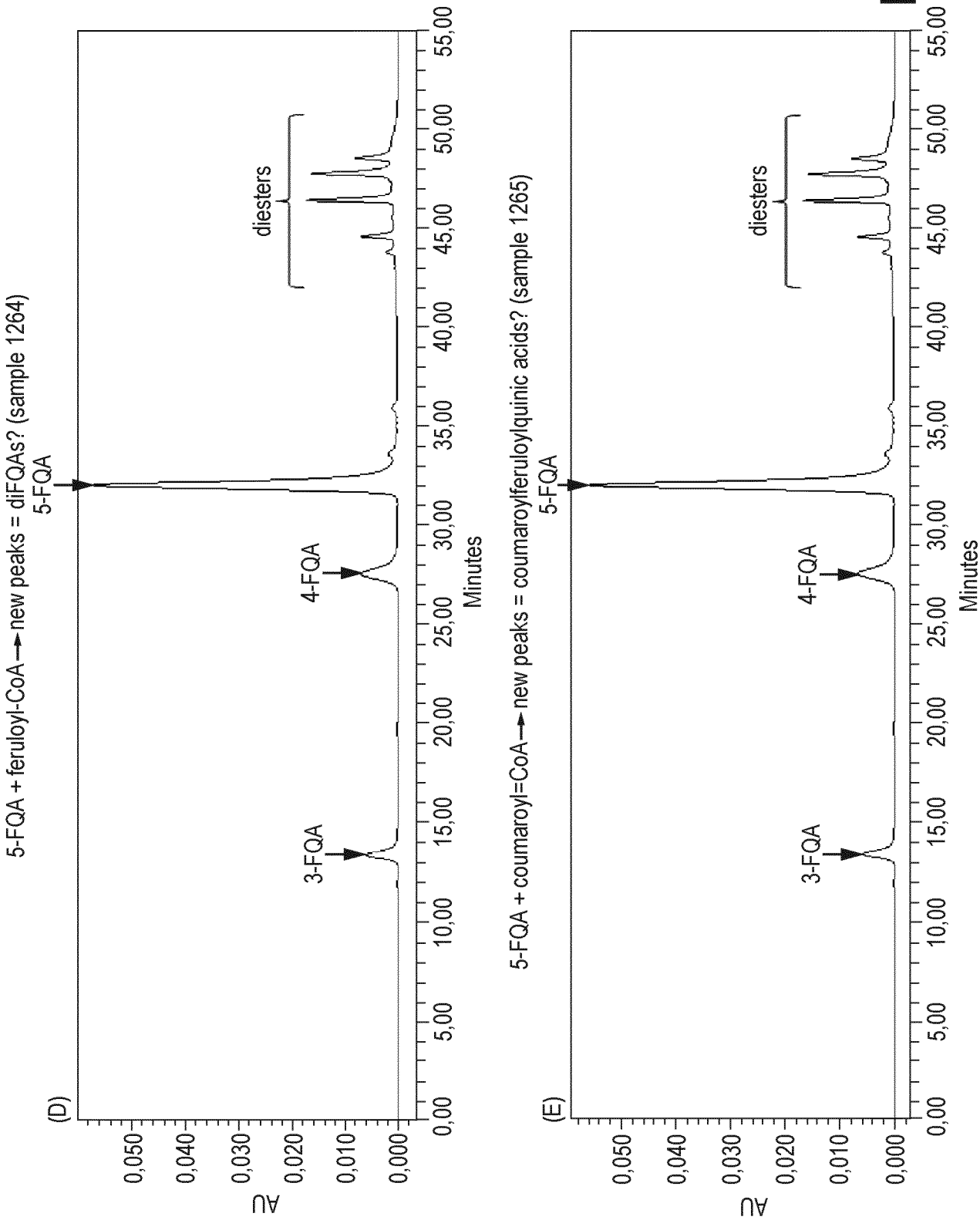

(D)
5-FQA + feruloyl-CoA → new peaks = diFQAs? (sample 1264)
5-FQA
4-FQA
3-FQA
diesters
AU
0,000
0,010
0,020
0,030
0,040
0,050
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
(E)
5-FQA + coumaroyl=CoA → new peaks = coumaroylferuloylquinic acids? (sample 1265)
5-FQA
4-FQA
3-FQA
diesters
AU
0,000
0,010
0,020
0,030
0,040
0,050
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 38 Cont'd

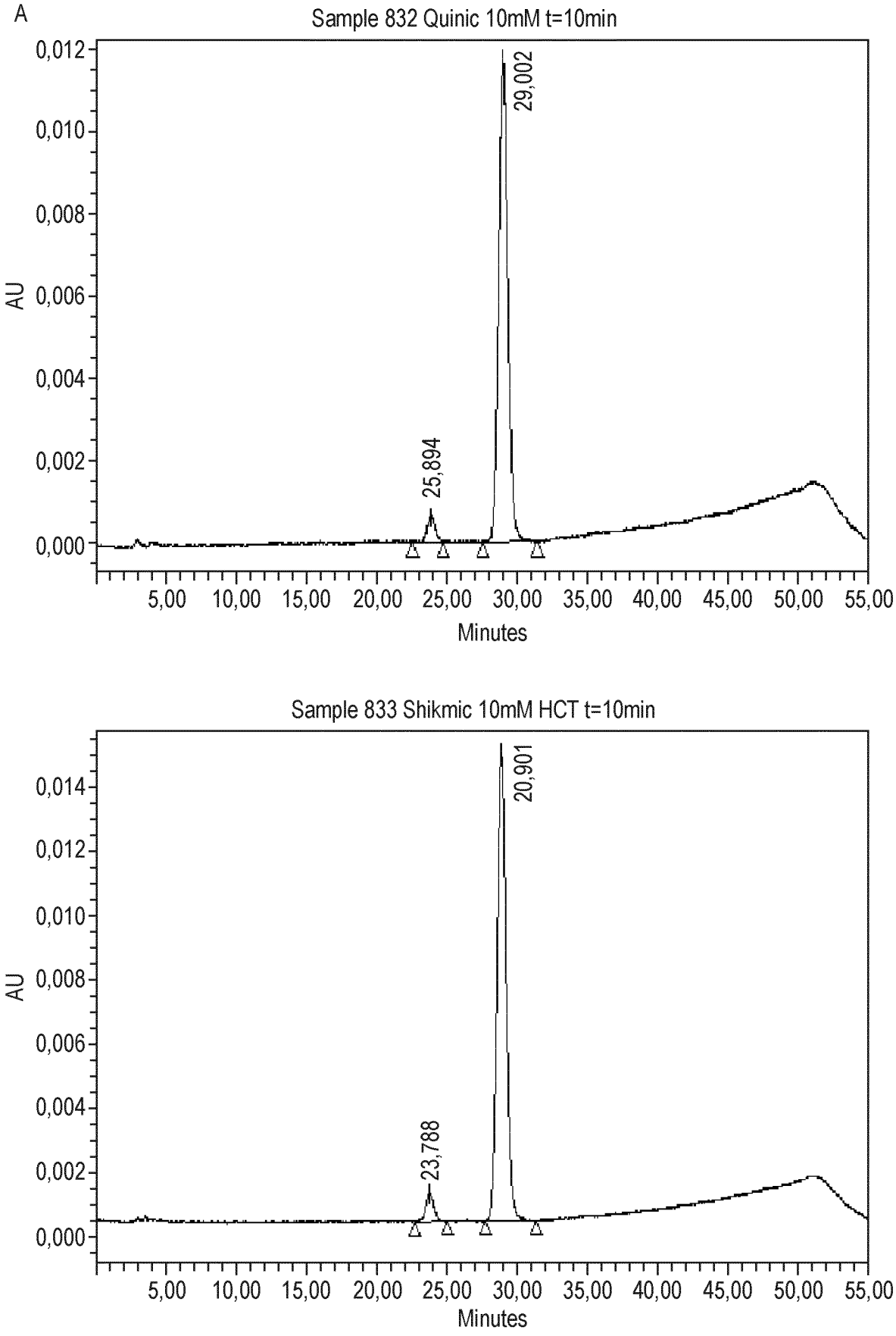
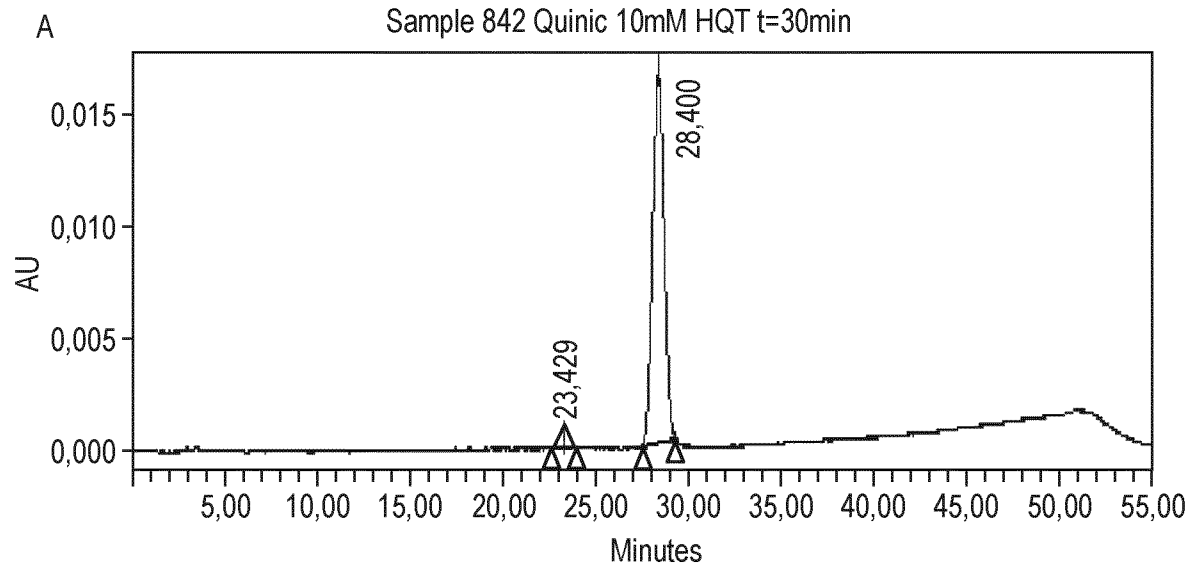

A
Sample 832 Quinic 10mM t=10min
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
29,002
25,894
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
Sample 833 Shikmic 10mM HCT t=10min
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
20,901
23,788
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 39


A
Sample 842 Quinic 10mM HQT t=30min
0,015
0,010
0,005
0,000
AU
28,400
23,429
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

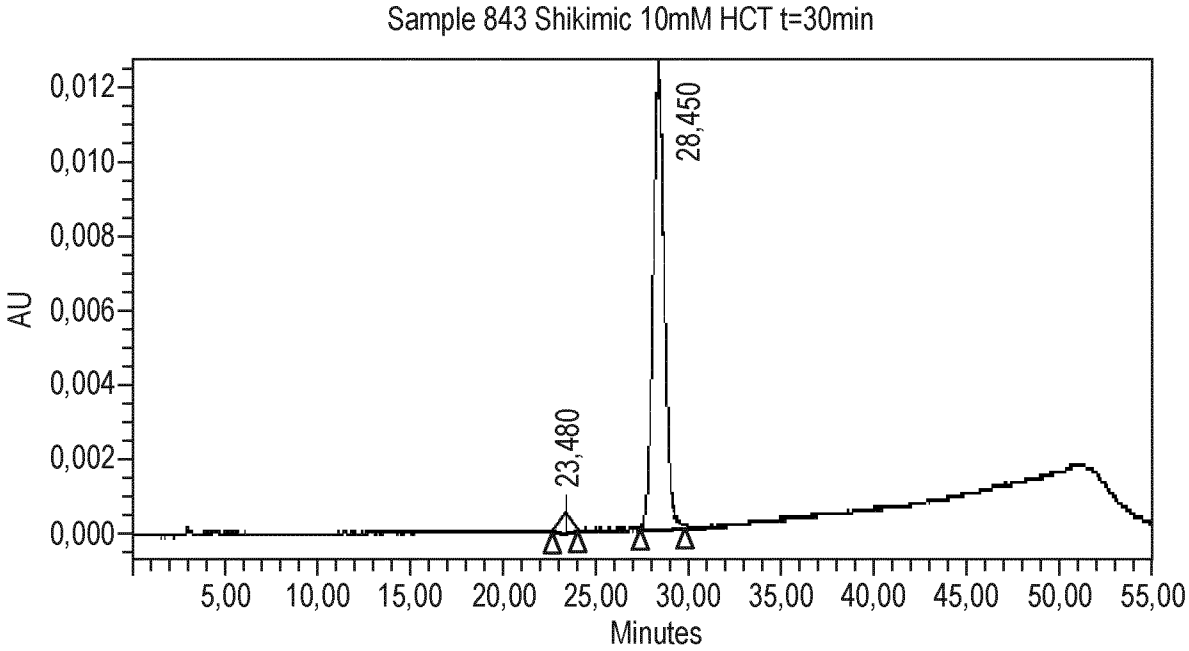

Sample 843 Shikimic 10mM HCT t=30min
0,012
0,010
0,008
0,006
0,004
0,002
0,000
AU
28,450
23,480
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 39 Cont'd

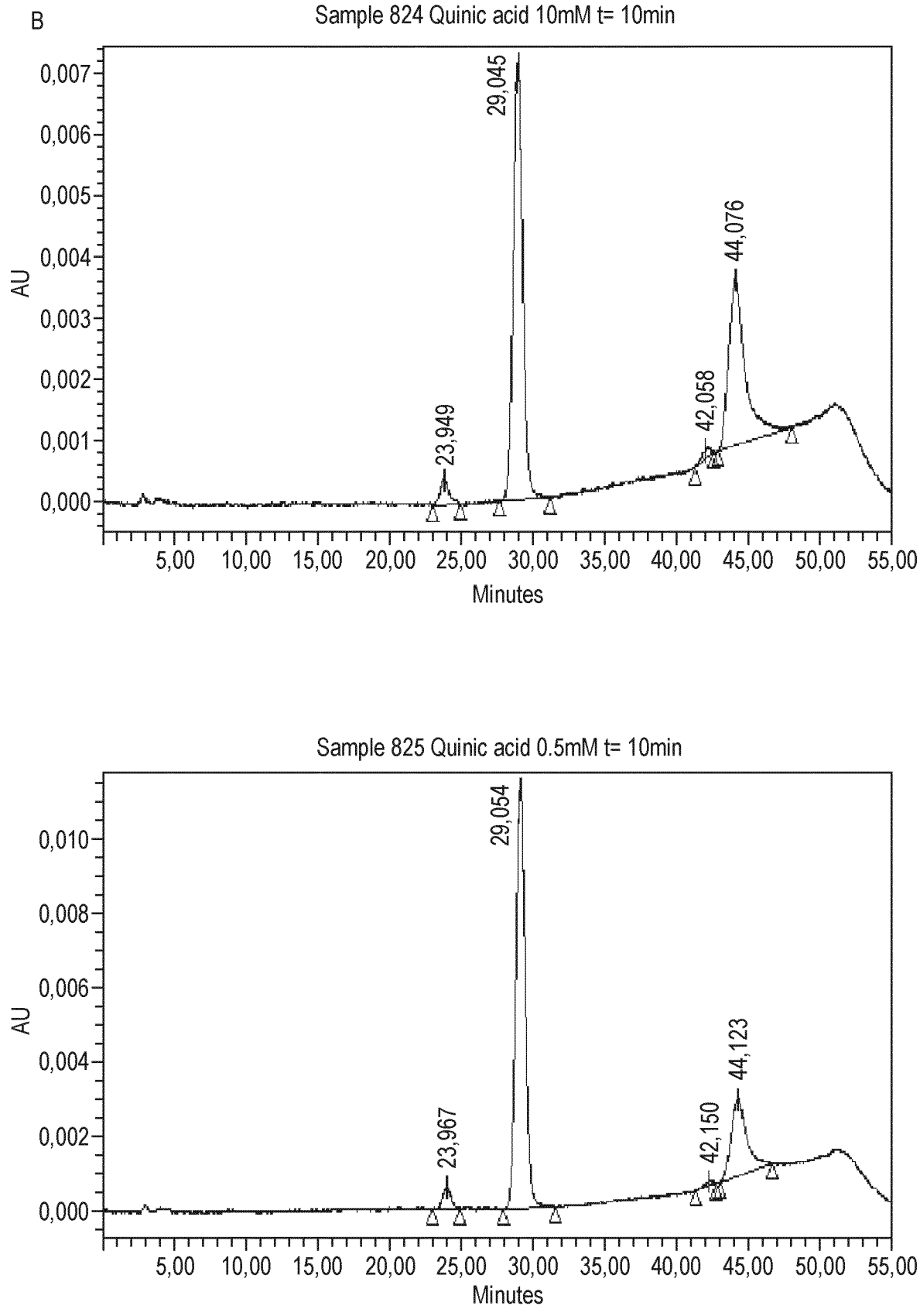

B
Sample 824 Quinic acid 10mM t= 10min
AU
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
29,045
44,076
42,058
23,949
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
Sample 825 Quinic acid 0.5mM t= 10min
AU
0,010
0,008
0,006
0,004
0,002
0,000
29,054
44,123
42,150
23,967
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 39 Cont'd

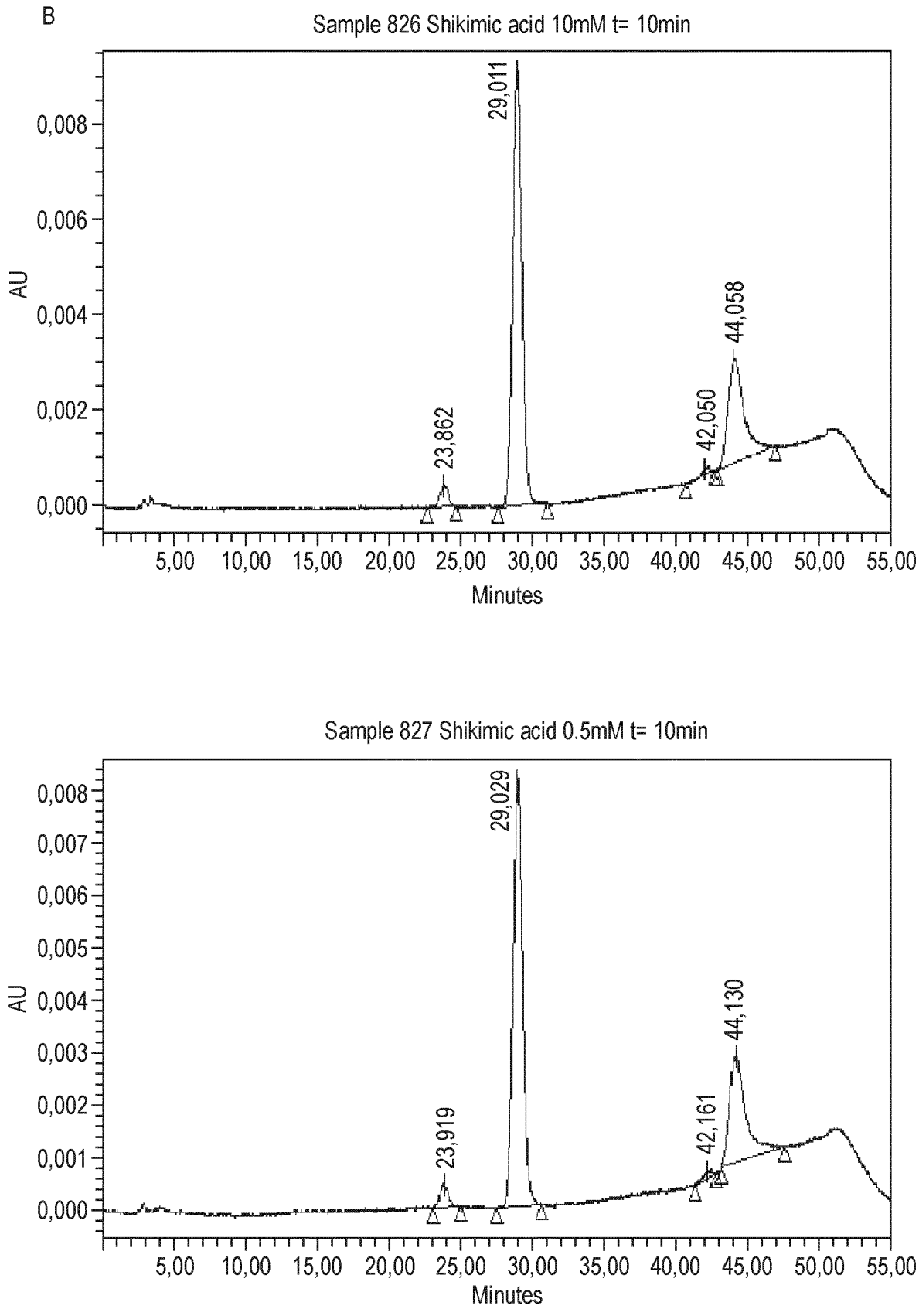
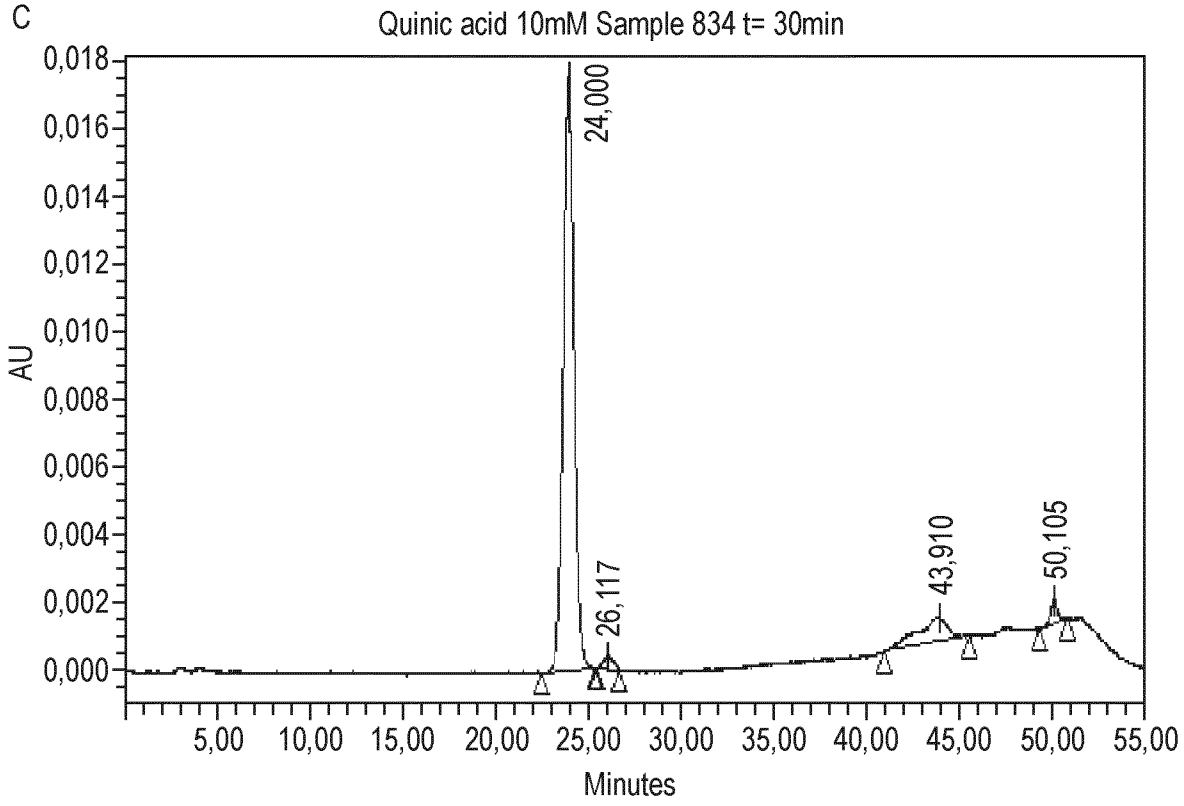

B
Sample 826 Shikimic acid 10mM t= 10min
AU
0,008
0,006
0,004
0,002
0,000
29,011
44,058
42,050
23,862
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes
Sample 827 Shikimic acid 0.5mM t= 10min
AU
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
29,029
44,130
42,161
23,919
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 39 Cont'd

C



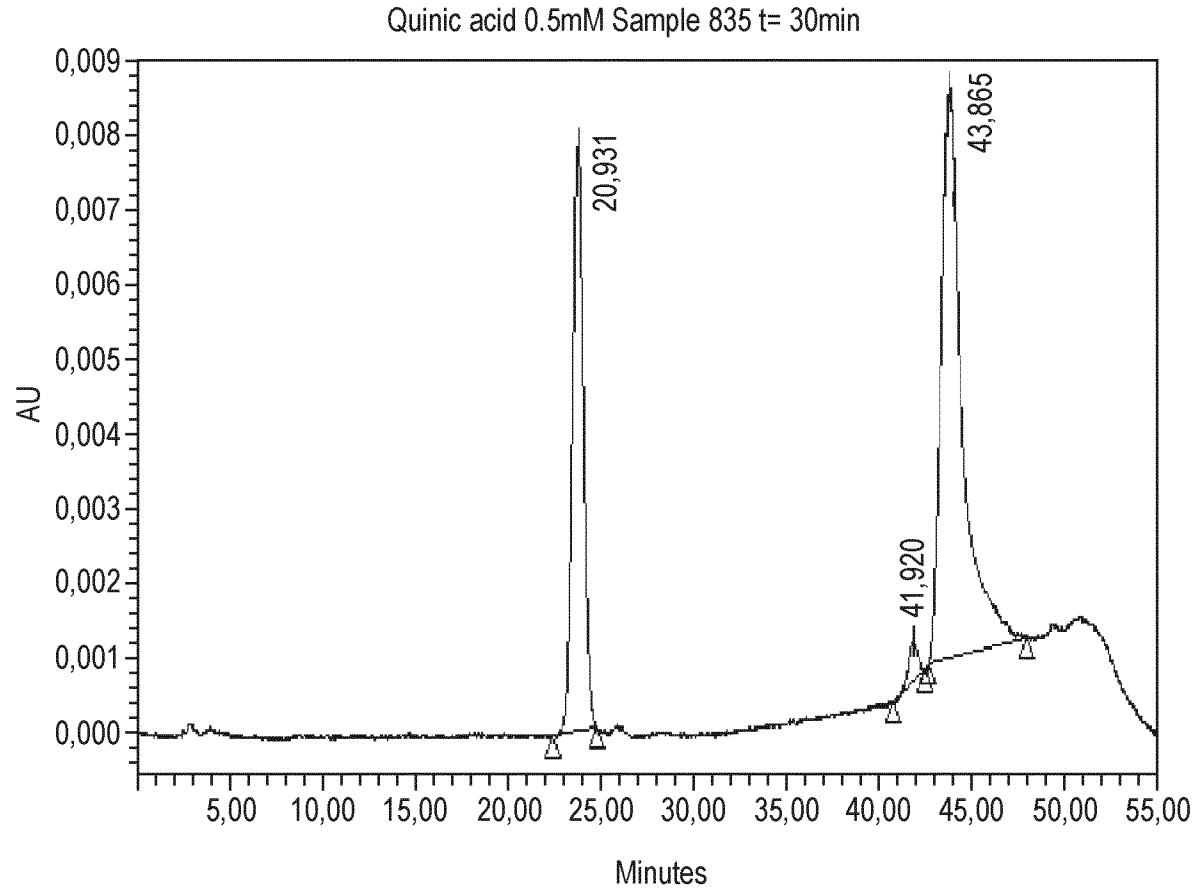
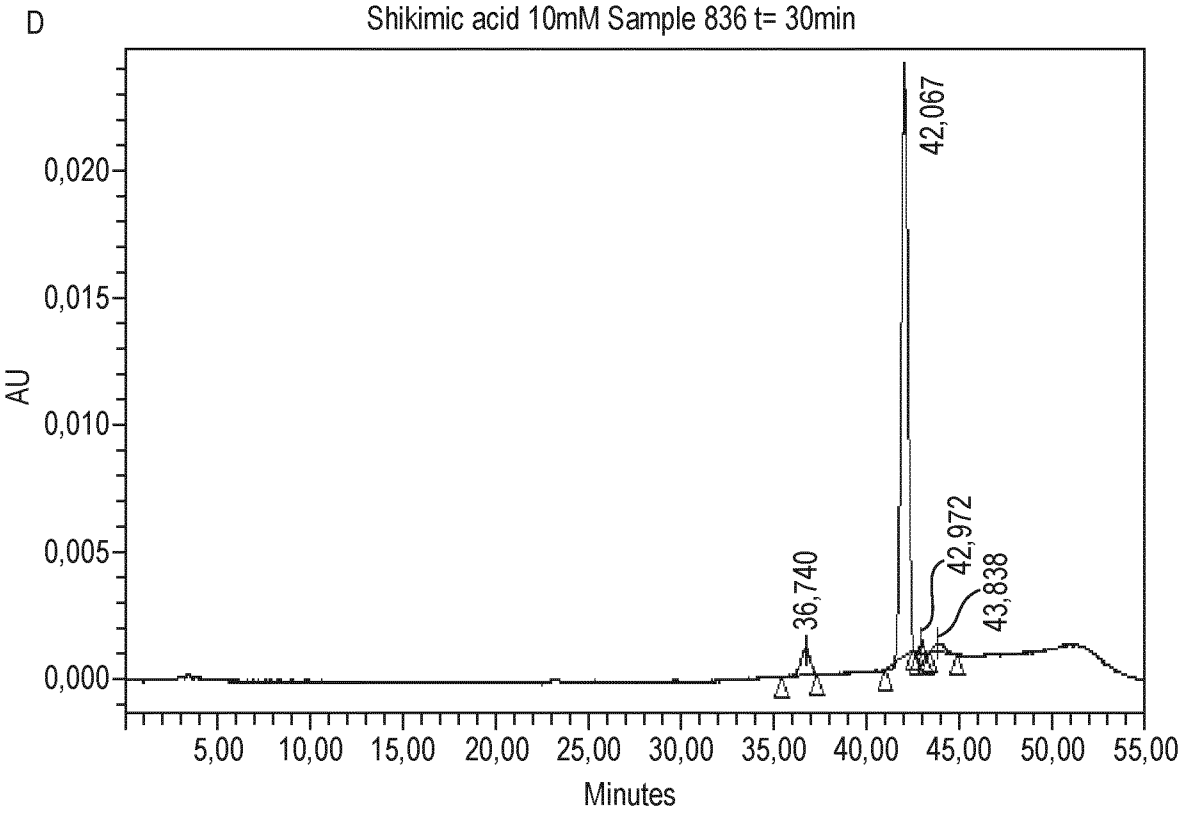


FIG. 39 Cont'd

D



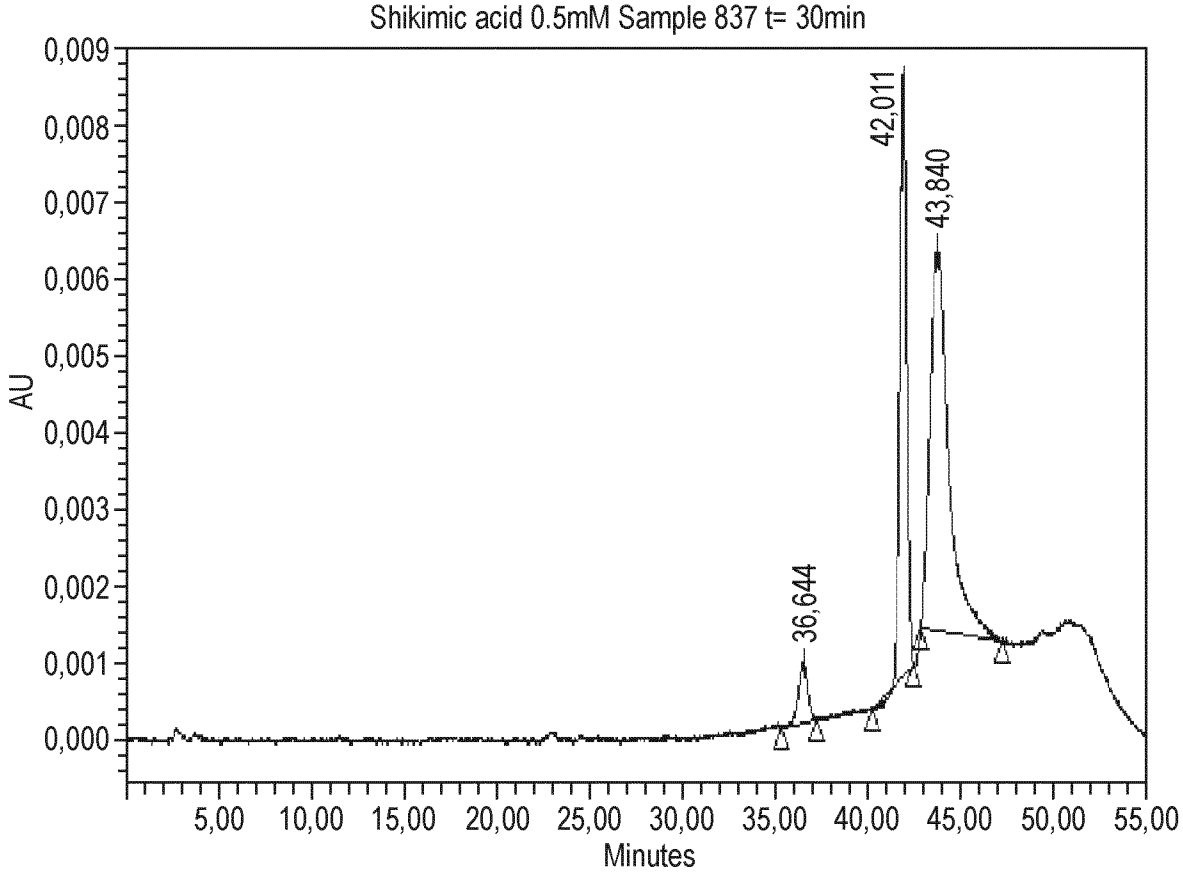
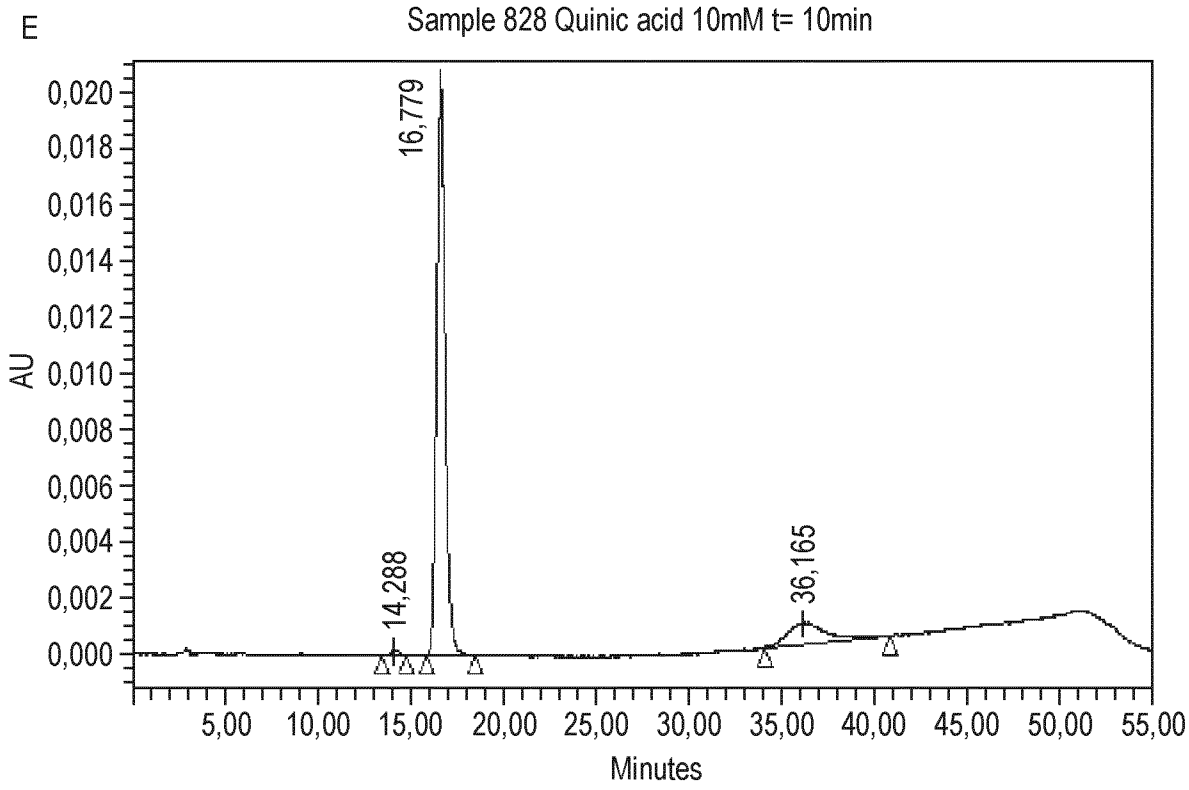


FIG. 39 Cont'd

E



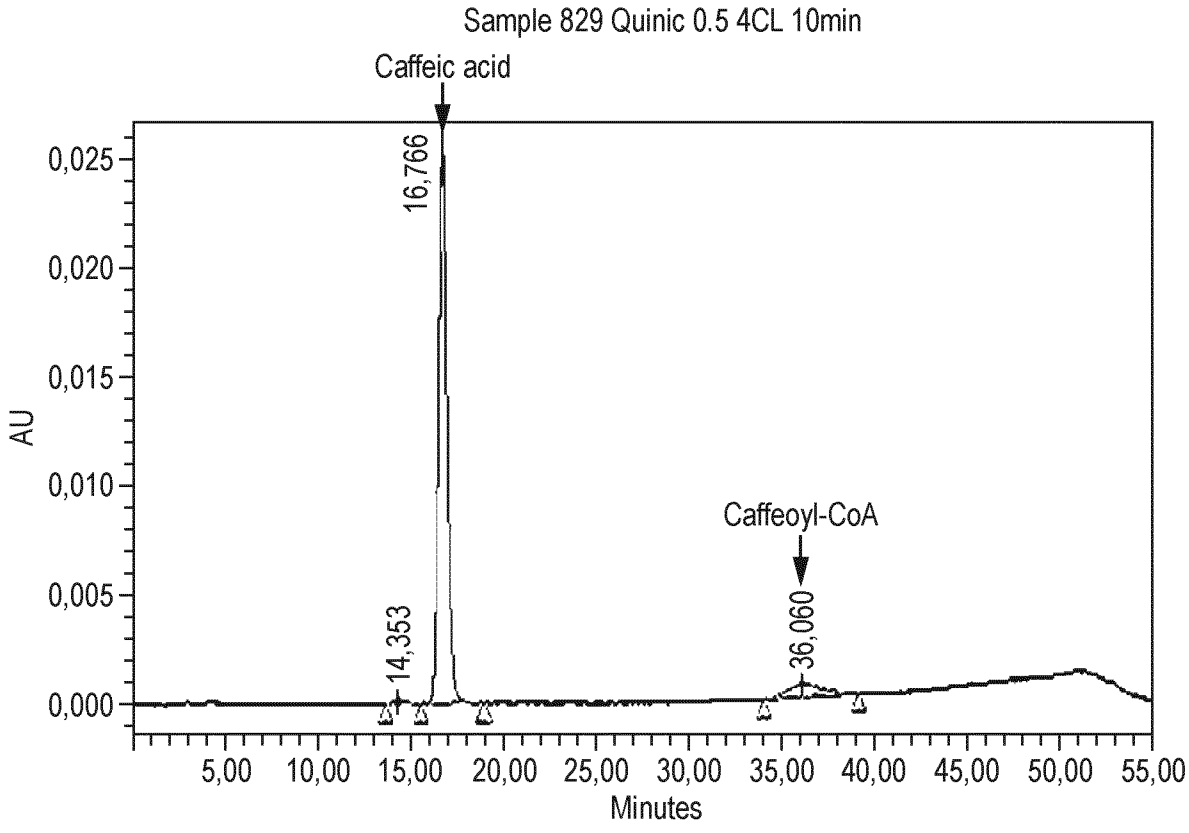
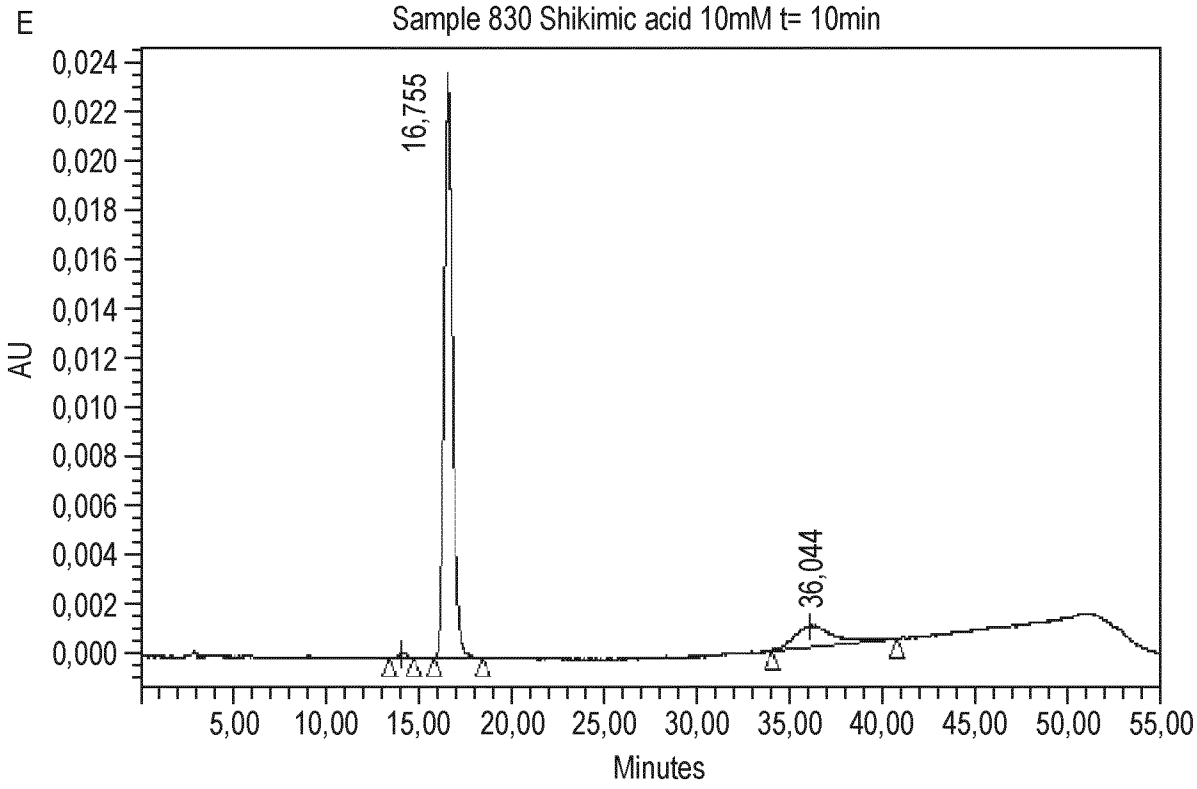


FIG. 39 Cont'd

E



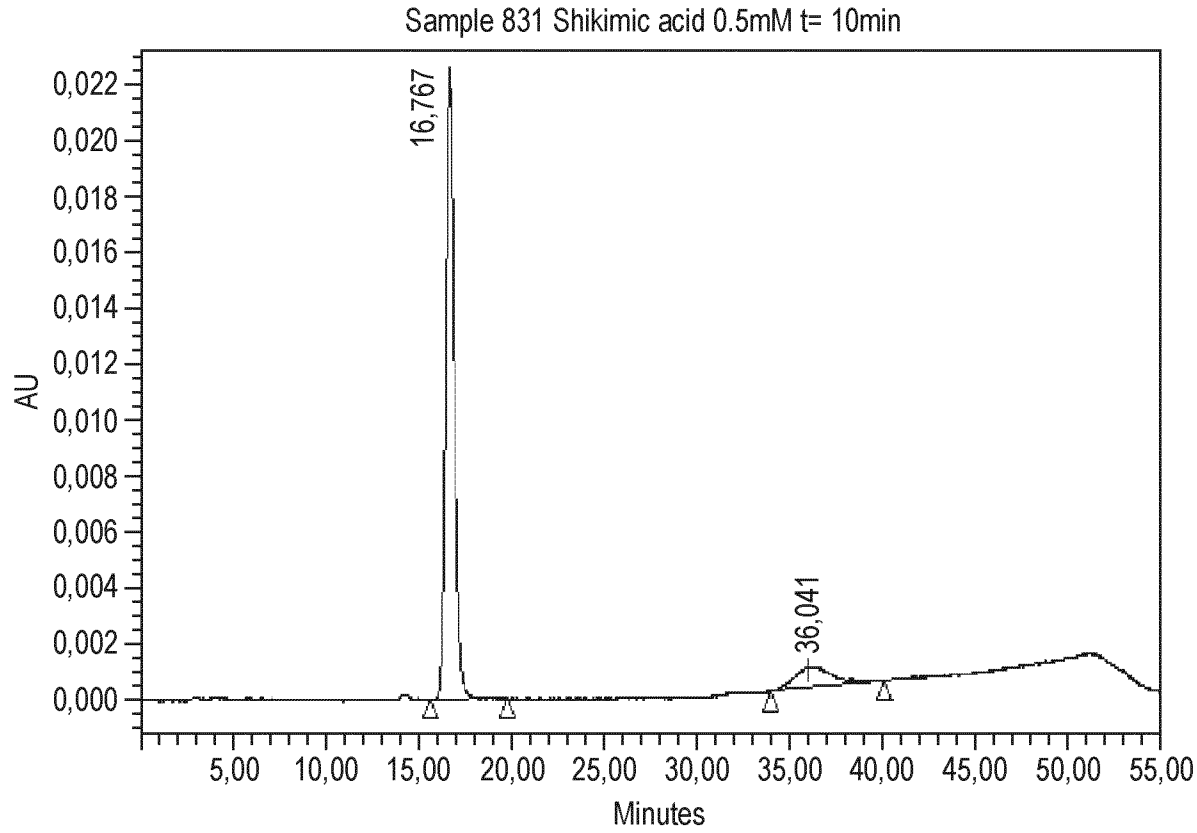
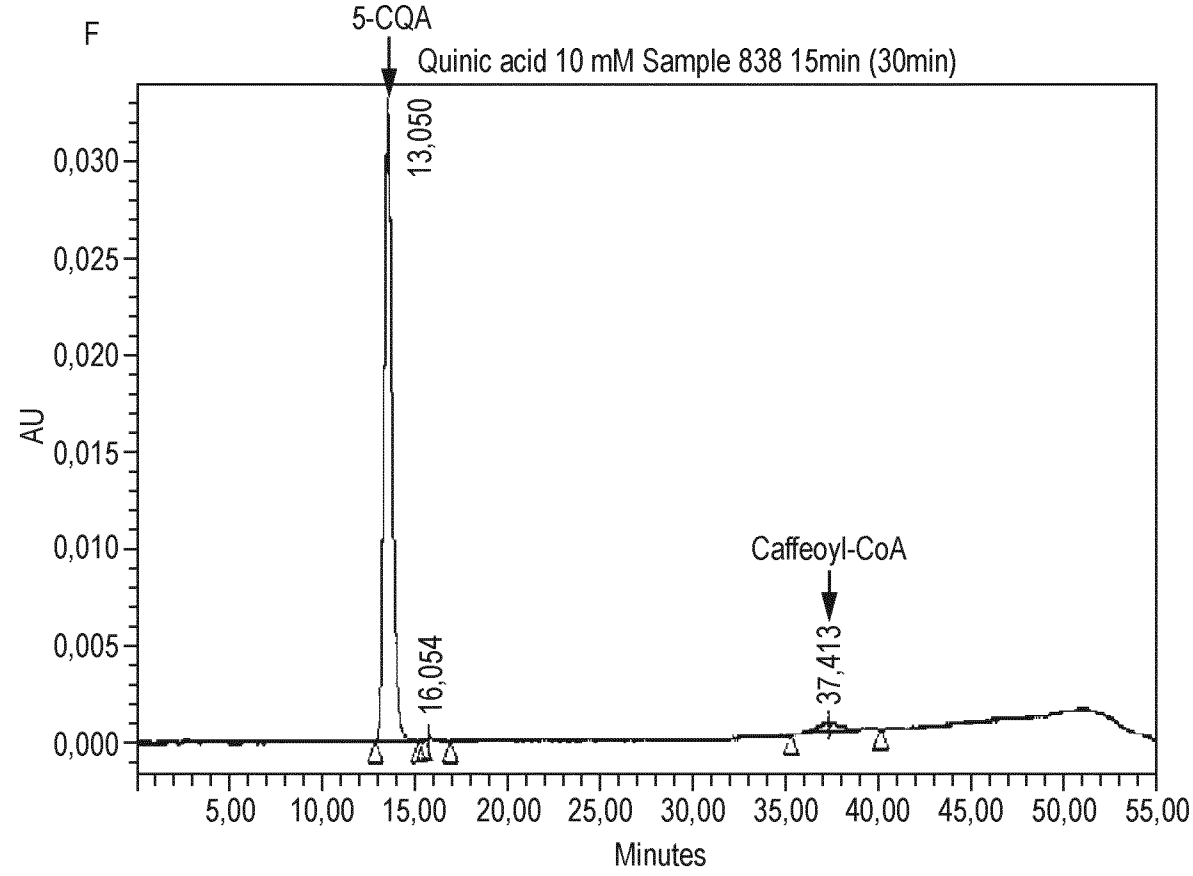


FIG. 39 Cont'd


F
5-CQA
Quinic acid 10 mM Sample 838 15min (30min)
13,050
16,054
Caffeoyl-CoA
37,413
AU
0,030
0,025
0,020
0,015
0,010
0,005
0,000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

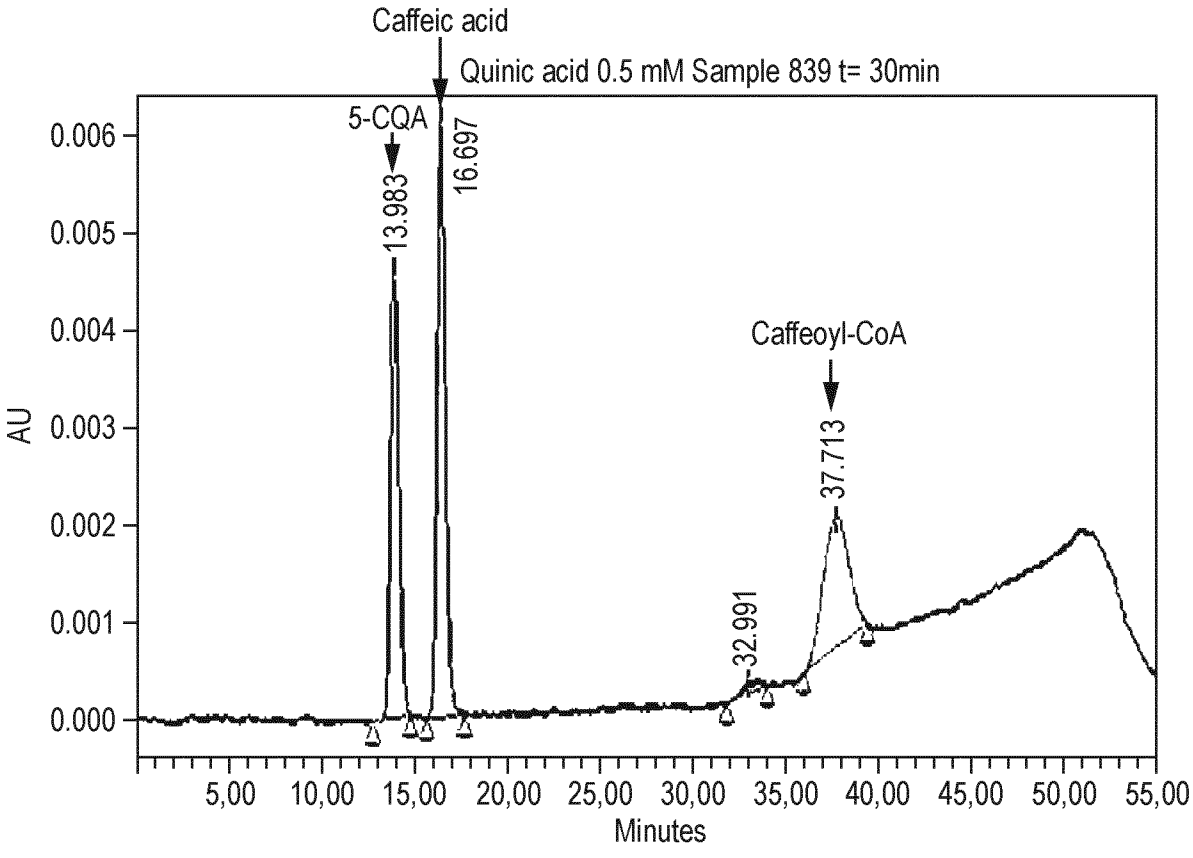

Caffeic acid
Quinic acid 0.5 mM Sample 839 t= 30min
5-CQA
13.983
16.697
Caffeoyl-CoA
37.713
32.991
AU
0.006
0.005
0.004
0.003
0.002
0.001
0.000
5,00 10,00 15,00 20,00 25,00 30,00 35,00 40,00 45,00 50,00 55,00
Minutes

FIG. 39 Cont'd

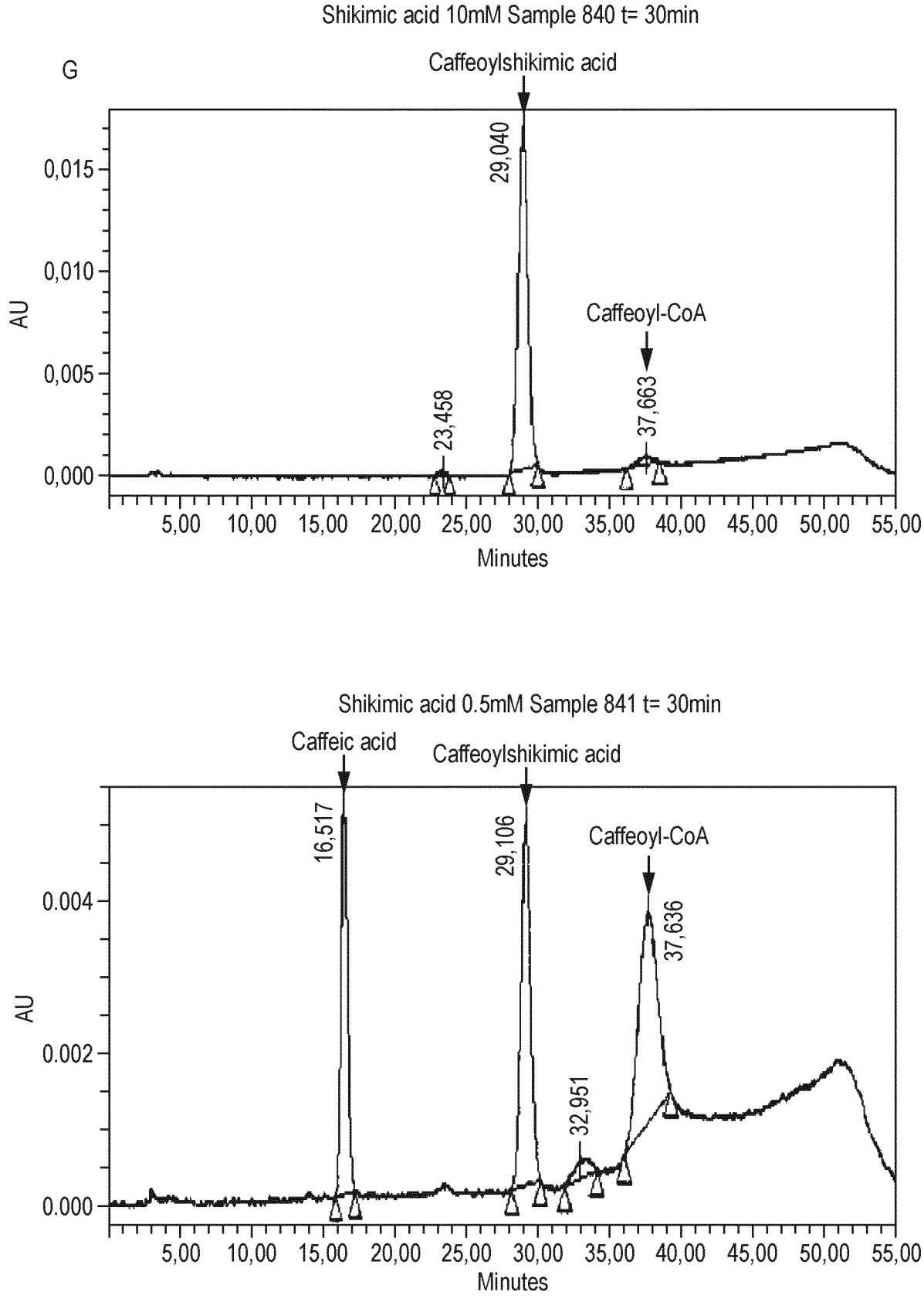

Shikimic acid 10mM Sample 840 t= 30min
G
Caffeoylshikimic acid
29,040
Caffeoyl-CoA
37,663
23,458
AU
0,015
0,010
0,005
0,000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes
Shikimic acid 0.5mM Sample 841 t= 30min
Caffeic acid
Caffeoylshikimic acid
16,517
29,106
Caffeoyl-CoA
37,636
32,951
AU
0.004
0.002
0.000
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

FIG. 39 Cont'd

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/054506

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/415 C12N15/82 A01H5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/044751 A2 (CORNELL RES FOUNDATION INC [US]; NESTEC SA [CH]; LEPELLEY MAUD [FR]; C) 19 April 2007 (2007-04-19) page 19, last paragraph - page 20, paragraph 1 page 25, paragraph 2; sequences 9,11 & DATABASE Geneseq [Online] 14 June 2007 (2007-06-14), "Robusta coffee HCT protein.", retrieved from EBI accession no. GSP:AFU68907 Database accession no. AFU68907 sequence 9 -/--	1,3-5,9, 10,14,15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2012	10/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Burkhardt, Peter

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DATABASE Geneseq [Online] 14 June 2007 (2007-06-14), "Robusta coffee HQT protein.", retrieved from EBI accession no. GSP:AFU68909 Database accession no. AFU68909 sequence 11 -----	
A	WO 2007/044992 A2 (CORNELL RES FOUNDATION INC [US]; NESTEC SA [CH]; LEPELLEY MAUD [FR]; C) 19 April 2007 (2007-04-19) page 23, last paragraph; sequence 26 & DATABASE Geneseq [Online] 26 July 2007 (2007-07-26), "C. canephora 4-coumarate:CoA ligase (4CL2)", retrieved from EBI accession no. GSP:AFU40754 Database accession no. AFU40754 sequence 26 -----	1,3-5,9, 10,14,15
A	LEPELLEY ET AL: "Chlorogenic acid synthesis in coffee : An analysis of CGA content and real-time RT-PCR expression of HCT, HQT , C3H1, and CCoAOMT1 genes during grain development in C. canephora", PLANT SCIENCE, ELSEVIER IRELAND LTD, IE, vol. 172, no. 5, 9 February 2007 (2007-02-09), pages 978-996, XP022016080, ISSN: 0168-9452, DOI: 10.1016/J.PLANTSCI.2007.02.004 the whole document -----	1,3-5,9, 10,14,15
A	NIGGEWEG RICARDA ET AL: "Engineering plants with increased levels of the antioxidant chlorogenic acid", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 22, no. 6, 1 June 2004 (2004-06-01), pages 746-754, XP009087941, ISSN: 1087-0156, DOI: 10.1038/NBT966 the whole document -----	1,3-5,9, 10,14,15
X	WO 02/100192 A1 (GIVAUDAN SA [CH]; CHIEN MINGIJEN [US]; HAEUSLER ALEX [CH]; VAN LEERSUM) 19 December 2002 (2002-12-19) page 2, paragraph 4 - paragraph 5; claims -----	11-13
	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHI ET AL: "Yeast-cell-based microencapsulation of chlorogenic acid as a water-soluble antioxidant", JOURNAL OF FOOD ENGINEERING, BARKING, ESSEX, GB, vol. 80, no. 4, 18 January 2007 (2007-01-18), pages 1060-1067, XP005834550, ISSN: 0260-8774, DOI: 10.1016/J.JFOODENG.2006.06.038 the whole document -----	11-13

4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/054506

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4, 5, 10-13(completely); 1, 3, 9, 14, 15(partially)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 4, 5, 10(completely); 1, 3, 9, 14, 15(partially)

   relating to a HCT from Coffea and products and methods comprising said HCT

   ---

2. claims: 6-8(completely); 1, 3, 9, 14, 15(partially)

   relating to a HQT from Coffea and products and methods comprising said HQT

   ---

3. claims: 2(completely); 1(partially)

   relating to a 4CL from Coffea and products and methods comprising said 4CL

   ---

4. claims: 11-13

   relating to a food product comprising one or more exogenous chlorogenic of dactylifric acid species

   ---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/054506

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007044751 A2	19-04-2007	AU 2006302107 A1	19-04-2007
		BR PI0616979 A2	05-07-2011
		CA 2625350 A1	19-04-2007
		CN 101326194 A	17-12-2008
		EC SP088355 A	30-06-2008
		EP 1940868 A2	09-07-2008
		US 2009158456 A1	18-06-2009
		WO 2007044751 A2	19-04-2007
WO 2007044992 A2	19-04-2007	AU 2006299782 A1	19-04-2007
		EP 1937803 A2	02-07-2008
		JP 2009511071 A	19-03-2009
		US 2010037357 A1	11-02-2010
		WO 2007044992 A2	19-04-2007
WO 02100192 A1	19-12-2002	AT 305233 T	15-10-2005
		BR 0210372 A	10-08-2004
		CA 2446194 A1	19-12-2002
		CN 1516553 A	28-07-2004
		DE 60206378 T2	22-06-2006
		EP 1399034 A1	24-03-2004
		ES 2247339 T3	01-03-2006
		JP 2004528050 A	16-09-2004
		JP 2010075183 A	08-04-2010
		MX PA03011043 A	19-03-2004
		US 2003003212 A1	02-01-2003
		US 2004213881 A1	28-10-2004
		WO 02100192 A1	19-12-2002